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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

Workshop on
Antimetabolites and the Central Nervous System

Memorial Sloan-Kettering Cancer Center

Editors: William R. Shapiro
Bipin M. Mehta
Dorris J. Hutchison

February 2–3, 1976
New York, NY

Supported by Lederle Laboratories, Division of American Cyanamid; Burroughs Wellcome Co; The Upjohn Company; Memorial Sloan-Kettering Cancer Center; and the National Cancer Institute.

Introduction: Antimetabolites and Central Nervous System Neoplasia[1]

William R. Shapiro,[2,*] Bipin M. Mehta,[3] and Dorris J. Hutchison[4,5]

The use of antimetabolites as chemotherapeutic agents originated when Woods suggested that the chemotherapeutic properties of sulfanilamide were due to competition with the structurally related metabolite, *p*-aminobenzoic acid (1). The antagonism between the metabolites and their structural analogs has been demonstrated to affect not only living organisms, but also isolated enzyme systems. Such antagonistic reactions have also been used to elucidate the pathways of metabolic reactions and have led to the design and synthesis of new drugs for use experimentally and in clinical practice. Thus, as a consequence of the studies describing the structure of folic acid (2) and some of its biologic activities, analogs of folic acid were prepared and their activities determined. Similarly, studies on antimetabolite activities of purine and pyrimidine analogs were also undertaken (3).

The first successful use of an antimetabolite in the treatment of acute leukemia in childhood was reported by Farber and his associates (4). Since then the folic acid antagonist methotrexate, a pyrimidine analog cytosine arabinoside, and several other antimetabolites have been used in the treatment of acute leukemias and lymphomas (5–9).[6] Although the therapeutic effectiveness of methotrexate was long known, the problems encountered with the occurrence of resistance at low doses and the toxicity at high doses hampered the development of a successful therapeutic regimen. Interest in methotrexate was revitalized after Goldin et al (10–12) observed that the therapeutic index of methotrexate for L1210 leukemia was enhanced by the use of leucovorin[7] given at definite time intervals. Recently, Djerassi et al (13–15) reported long-term remissions in childhood acute leukemia after administering large doses of methotrexate followed by frequent doses of leucovorin. Leucovorin, the most stable form of reduced folates (16,17), is used therapeutically to reverse the toxic effects of methotrexate (18,19). The historical aspects of the development of leucovorin are worth noting. A naturally occurring growth factor active for *Leuconostoc citrovorum* ATCC 8081 was described by Sauberlich and Baumann (20) and Broquist et al (21) and was later designated as citrovorum factor. Shive et al (22) and Brockman et al (23) reported biologic activity for the same organism in a synthetic reaction mixture derived from folic acid. After the administration of folic acid, both 5-formyltetrahydrofolic acid and 10-formyltetrahydrofolic acid are found in urine, and both compounds are known as "citrovorum factors" since they are active in promoting the growth of *L. citrovorum* ATCC 8081. The chemistry of leucovorin (17) and its isolation in diastereoisomers have been described by Cosulich et al (24), and the relationship of folinic acid and *L. citrovorum* factors is described by Bardos et al (25). Thus, leucovorin, also called folinic acid, is *d,l*-5-formyltetrahydrofolic acid, and the commercially available calcium salt of leucovorin is half as active, on a molar basis, as the naturally occurring citrovorum factors (24). The interchangeable usage of the terms leucovorin, citrovorum factor, and folinic acid is thus understandable; however, the term citrovorum factor should be

[1] Our special thanks for support of the Workshop on Antimetabolites and the Central Nervous System are extended to Lederle Laboratories, Pearl River, NY; Burroughs-Wellcome Co, Research Triangle Park, NC; The Upjohn Company, Kalamazoo, Mich; Memorial Sloan-Kettering Cancer Center, New York, NY; and *Cancer Treatment Reports*, National Cancer Institute, Bethesda, Md.

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[5] We are especially thankful to Miss Shaune Blair, Mrs. Alice Braun, Mrs. Carol D'Anella, Miss Joanne M. Gallo, Miss Ann L. Gisolfi, Mrs. Varsha B. Mehta, and Mrs. Madeline R. Tappert for their assistance in the various phases of the successful organization of the Workshop.

[*] *Reprint requests to:* William R. Shapiro, MD, Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

[6] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

[7] Leucovorin: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

reserved for the natural constituent active in promoting the growth of *L. citrovorum* ATCC 8081, now designated *Pediococcus cerevisiae* ATCC 8081 (26).

In the last 25 years, the therapeutic effectiveness of methotrexate in the treatment of acute leukemia of childhood (13–15,27), choriocarcinoma (28), and Burkitt's lymphoma (29) along with some other nonneoplastic conditions has been demonstrated. However, the treatment of neoplasms localized in brain and cerebrospinal fluid (CSF) is seriously hampered by the relative exclusion of chemotherapeutic agents from these areas because of the blood-brain and blood-CSF barriers (30). The development of a progressive symptomatic leukemia localized in the arachnoidal meninges of children with acute leukemia in complete bone marrow remission following methotrexate treatment suggested inadequate penetration of methotrexate in the central nervous system (CNS) (31). Whiteside et al administered methotrexate intracisternally into dogs and later pioneered in the use of intrathecal methotrexate in the therapy of CNS leukemia (32,33).

Methotrexate treatment of CNS leukemia in the 1960s had limited success (34–36), but two factors substantially altered the results in the overall prognosis of patients with leukemia: (*a*) the use of prophylactic therapy to the CNS (37), and (*b*) the introduction of indwelling intraventricular cannulas (Ommaya) (38). It is now established that for children with acute lymphoblastic leukemia, in addition to intensive systemic chemotherapy, prophylactic CNS radiation therapy (39), combination radiation therapy, and intrathecal methotrexate (40), or prophylactic intrathecal methotrexate alone (41), substantially reduce the incidence of meningeal invasion by tumor and prolong the survival of such patients. Similarly, the treatment of acute leukemia in adults is improved by prophylactic intrathecal chemotherapy (42) although the results are not as good as they are in children. Ommaya reservoirs permit better distribution of drugs administered intrathecally (43) and have been used as an adjunctive method for delivering drugs into patients who are at greater risk of developing meningeal leukemia (44) (eg, patients with initial peripheral wbc counts $> 25{,}000/mm^3$). Ommaya reservoirs have also permitted intensive investigation of drug distribution in the CNS of patients (43) and have been utilized in the therapy of brain tumors (45) and meningeal carcinomatosis (46).

Djerassi introduced the concept of high-dose methotrexate with leucovorin rescue in the management of leukemia (13,14) and this concept has been extended by Jaffe et al (47), Rosen et al (48), and others (49) to the treatment of osteogenic sarcoma in children. Its potential value in the therapy of other tumors is being investigated (50). Most recently, high-dose methotrexate has been used in the prophylaxis and therapy of children with acute lymphoblastic leukemia, as described in this Workshop.

Antimetabolites, especially methotrexate, have been tried in the treatment of adults and children with malignant brain tumor with varying results (51). In most cases, an effort was made to deliver drugs in higher concentration into the CNS, either by infusion into the carotid artery or by intrathecal administration. Current chemotherapy of malignant brain tumors has been advanced by the demonstrated effectiveness of the nitrosoureas (52), but to date no cell-cycle-specific agent has been found to be effective. The blood-brain barrier excludes most antimetabolites and it is this problem which prompted this Workshop.

We attempted to review the state of the art of the use of antimetabolites in systemic disease and especially in CNS disease, and the Workshop was organized to cover four separate issues:

(1) The action of antimetabolites at the cellular level. This included the kinetics of entry, the interconversion among the folates, intracellular antimetabolite concentrations and enzyme binding (especially between methotrexate and dihydrofolate reductase), and the relationship between intracellular drug action and leucovorin "rescue."

(2) The distribution of antimetabolites, especially methotrexate and cytosine arabinoside, in and around the CNS. Of special interest was methotrexate entry into the brain from the CSF, entry into the brain from the blood after high-dose administration, and distribution of leucovorin and its main metabolite, 5-methyltetrahydrofolate, in and about the CNS after systemic administration.

(3) The value of antimetabolites in the treatment of meningeal leukemia, meningeal carcinomatosis, and brain tumors. Since there exists comparatively greater experience with high-dose methotrexate therapy for non-CNS tumors, the Workshop also reviewed the therapy of leukemia (especially meningeal leukemia), osteogenic sarcoma, and several other solid tumors. Of special interest was the use of antimetabolites in the therapy of children with malignant brain tumors and adults with meningeal carcinomatosis.

(4) CNS toxicity from methotrexate, the relationship of such toxicity to methotrexate doses, and route of administration, prior radiation therapy, and mechanical factors such as ventricular obstruction.

The increasing interest in the antimetabolites, especially high-dose methotrexate, in chemotherapy of cancer has been demonstrated by recent conferences concerned with the pharmacology and cellular biology of the action of methotrexate (53). Our aim in this Workshop however, was to look at specific problems and potential solutions to the use of antimetabolites in tumors in and of the CNS. This is a new field, and the conference raised many more questions than it answered. Lengthy discussions were generated, and ideas presented which might be amenable to experimental approach. Specific problems of toxicity were reviewed. The obvious need to correlate laboratory with clinical investigation was again recognized and, as noted in the concluding remarks by Dr. Lewis Thomas, the increasing complexities of this subject will require even closer cooperation between the clinician and the laboratory investigator. We hope the results from this conference will spur such cooperation.

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Looking Backward While Going Forward

C. Chester Stock [1,*]

An appropriate subtitle for my introductory remarks, if I may use the vernacular of advertising, would be "You've come a long way baby." Truly the antimetabolite concept has been exploited remarkably. Particularly impressive have been the developments with the antifolates and the antinucleic acid precursors for the therapy of cancer.

I welcome the opportunity to look backward briefly on the developments in antimetabolites and possibly to set the stage for the explorations and advances presented in this Workshop. Part of my appreciation in being able to review briefly some of the history is that I have occasionally been a fortunate participant in a few developments and always an interested and at times an excited observer of nearly all of the developmental period of antimetabolites.

I refer to the developmental period, for as Woolley (1) noted in his book there were early signs, numerous and varied, pointing the way to the antimetabolite concept: the inhibition of enzymes by substrate analogs (2,3); the inhibition of antibody reactions with antigens by the related haptens (4); and the production of metabolic and enzyme deficiencies by such materials as ethionine (5) and 4-hydroxycoumarin (6).

I believe there has been general agreement that it was the series of events initiated by Domagk's (7) discovery of therapeutic activity of Prontosil that clearly brought forward the antimetabolite concept. I recall the great interest with which all of us on a ward round at the Johns Hopkins Hospital (Baltimore, Md) in the latter part of 1936 heard Perry Long tell of the successful cure by Prontosil of a patient seriously infected with a hemolytic streptococcus. The subsequent essential steps in the development of antimetabolites were those by Fourneau and associates (8) which showed that sulfanilamide was the chemotherapeutically active moiety of Prontosil and Woods' (9) observation that *p*-aminobenzoic acid (PABA) blocked the bacteriostatic action of sulfanilamide and related sulfonamides in a competitive way. Woods' (9) prediction that PABA would be found an essential biologic compound was soon verified by observations that it was an essential nutrient for several species (10,11) and by recognition that it was an integral part of folic acid, as reported by the Lederle Laboratories group (12).

While these developments came just before and early in World War II, many efforts were made to exploit them and Fildes' (13) suggestion in 1940 that useful chemotherapeutic agents against infectious diseases might be produced by altering the structure of some other vitamin or metabolite. From 1942 to 1947, a considerable number of antagonists were found (14). While those years might have been even more productive had not the war intervened, it ultimately may have provided a stimulus through the recognition of the wartime successes of the sulfonamides and penicillin. My own wartime activities as a technical aide for several Office of Scientific Research and Development and National Research Council committees concerned with chemical warfare agents, insecticides, and rodenticides provided frustrations at being out of the laboratory while reading the accumulating information on antimetabolites and seeing in our program the effectiveness of the rodenticide, sodium fluoroacetate, an obvious candidate for classification as an antimetabolite.

During the war years various laboratories were working on the isolation of growth factors with a variety of names, which were based in part on the test organisms that defined the activities. These investigations ultimately converged in the identification of folic acid. At that same time studies were conducted by Lewisohn and associates (15,16) who reported that extracts of yeast and barley and preparations of *Lactobacillus casei* factor exhibited antitumor properties; however, Sugiura (17), working closely with Lewisohn's group, failed to confirm the findings with spontaneous mammary adenocarcinomas in mice. In this complicated situation Sugiura attributed the reported regressions to effects of the tumor biopsies while Lewisohn believed lack of confirmation was due to technical problems. In view of developing information on immunostimulations, one now may speculate that such effects may have been inconsistently involved in the studies with crude preparations. The report of the effect of folic acid preparations in animals had its parallel in

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TABLE 1.—Inhibition of growth of Sarcoma 180 by analogs of folic acid

Compound	Dose (mg/kg/day)	Effect*
Pteroyl triglutamic acid (Teropterin)	2000	–
Pteroyl glutamic acid (folic acid)	150	–
Pteroyl aspartic acid (*d*)	150	–
Pteroyl aspartic acid (*dl*)	75	–
4-Amino pteroyl aspartic acid	30	+
4-Amino-N^{10}-methyl pteroyl glutamic acid	1.5	+
4-Amino pteroyl glutamic acid	0.2	+

*+ = no development of tumor or growth to 25% average diameter of the controls; – = growth from 75% to 100% diameter of controls.

the report of the benefit of folic acid or its congener in human leukemia. This was paradoxical in view of later observations on the antifolates and reports on folic acid at an earlier meeting (18) in May 1946. Particularly after hearing about the beneficial effects of folic acid upon anemia and leukopenia it was easy for me, as it must have been for others, to hold the conviction that an antifolate should be effective against leukemia. Little did we know that such compounds were then no doubt in the notebooks at Lederle, if not actually in the hands of Sidney Farber. Farber had a pioneering role in the use of antifolates in human leukemia and great excitement was generated by the report of his early successes with aminopterin (19).

When we were able to obtain antifolates beyond the X-methyl derivative, we were pleased to see that our test tumor, mouse Sarcoma 180, responded to the antifolates (table 1) (20). Possibly as a result of changes in the tumor through the years, Sirotnak et al (21) now find it inadequately responsive unless treatment is based upon some of the interesting data they have collected. We also observed the apparently better results with methotrexate (22) compared with aminopterin against Sarcoma 180. Table 2 shows the results for methotrexate as we presented them in 1949. Although, at that time, methotrexate definitely impressed us as being the superior drug, I am not certain that a statistical analysis would confirm our impression.

There have been many reports on the antitumor and other biologic activities of the antifolates and they are too numerous to review here. These included the extensive observations on resistances developed by leukemias to antifolates and other antimetabolites studied so actively by Hutchison and Schmid (23) and, pertinent to this meeting, the blocking of methotrexate toxicity and antitumor activity by citrovorum factor by Burchenal et al (24). Antifolates with other heterocyclic rings have also been the subject of study by a number of investigators (25–28).

The years that followed brought other antimetabolites with antitumor activity. The antibiotics azaserine and DON (29,30) developed with Parke, Davis and Co, though failures in the clinic, I believe merit more extensive investigation. In a cooperative program with Hitchings and Elion (31) we were able to detect initially the activity of 6-mercaptopurine in Sarcoma 180. Later, 6-thioguanine was also found to be active (32). Synthesis of theoretically attractive purine analogs has not always provided useful agents. 8-Azaguanine and 4-aminopyrazolopyrimidine are two agents whose toxicity to normal tissue demonstrated the early anticipated problem that some antimetabolites would be failures in animal or human cancer due to the impact of the antimetabolite action on normal host cells. This appeared to be true of a series of 2,4-diaminopyrimidines in initial studies with Hitchings, but new information to provide a basis for their more effective utilization may give them new utility.

Developments came in the antipyrimidine area with Heidelberger's (33) 5-fluorouracil in the latter 1950s and with cytosine arabinoside from the Upjohn group (34). Cytosine arabinoside and related compounds will comprise a significant portion of this Workshop.

Some of the ways metabolite deprivation may be achieved are by the use of antimetabolites, dietary exclusion, and enzymatic destruction. The interest stemming from the use of L-asparaginase has extended to studies of folate-degrading enzymes which

TABLE 2.—Effect of different doses of methotrexate on the growth of Sarcoma 180

Dose (mg/kg/day)	No. of groups of 5 mice	No. of mice	Deaths during injection period		Effect on tumor*			
			No.	%	Inconclusive	Marked inhibition	Moderate	None
2.5	6	30	14	46.6	2	4	0	0
2.0	15	75	14	18.6	2	9	4	0
1.5	31	155	11	7.1	0	16	11	4
1.0	8	40	2	5.0	0	1	4	3

*Tumor effects are expressed as the result obtained in each group of 5 mice. Thus, "2" indicates 2 groups of 5 mice each.

Albrecht et al (35) and Bertino et al (36,37) are pursuing.

The reports of this Workshop emphasize that we have embarked upon the next phase after demonstrating that a number of metabolites can be effective against certain forms of human cancer. We are now in the stage of the ultimate exploitation of the potentialities of the antimetabolite approach which will come through the diligent and intelligent application of the fundamental information which many investigators are actively gaining and applying to the clinic.

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Antifolates: In Vivo Kinetic Considerations

Daniel S. Zaharko[1,*] and Robert L. Dedrick[2]

SUMMARY

The action of antimetabolites in vivo involves the consideration of several interrelated factors. If the mechanism of biochemical action is understood, the next items of priority are whether the active form of the drug gets to the site of action, its concentration at this site, the time course of removal of active drug from this site, and how these kinetics relate to the biochemical inhibition. Studies of antifolates represent some of the approaches used to further understand antimetabolite action in vivo. The interrelationships of blood flow, mixing volumes of tissue compartments, membrane resistance, binding of drug, binding site formation, transport competition, and biochemical kinetics of rate-limiting steps, substrate competition, and salvage pathways are considered from an in vivo point of view.

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In the search for more successful treatment of neoplasms with chemotherapeutic agents there are basically two approaches: (*a*) attempts to find new drugs with unique or improved action, and (*b*) attempts to improve the effectiveness of drugs already proven to be useful clinically. Both approaches are important and should be conducted concurrently. The theme of this Workshop and this presentation particularly covers the latter approach.

Experimental studies both in vitro and in vivo can result in qualitative concepts and in some cases quantitative predictions. It is important to differentiate between these two and to understand the limitations and strengths of experimental systems so that experimental findings and presentations are interpreted in the proper perspective. We hope that clinical quantitative predictions are not made from experimental findings that are unique to the experimental system being studied. I think our bias towards the importance of the experiments we personally conduct occasionally makes all of us guilty of such extrapolation. Our studies and observations of findings with folate antagonists have re-emphasized to us the uniqueness of individual experimental systems. Our approach has been to further explore the reasons for uniqueness so that qualitative concepts may be more readily understood and interpreted in the framework of available quantitative data. We first got into this line of investigation by studying factors which contributed to interspecies differences in the pharmacokinetic behavior of drugs. We are currently concentrating on the kinetic responses of pharmacologic effect to drugs exhibited by different organs and tissues within the same animals. I would like to summarize some of our thoughts on these subjects.

Drug Delivery to the Site of Action

There are several important factors which determine the concentration of a drug at the site of action and its residence time at this site. One of the most obvious factors is whether the active form of the drug is being delivered. Since biotransformation is discussed in other papers in this issue, our comments on the subject will be brief. Few in vivo quantitative studies have been attempted which relate antimetabolite drug metabolism to pharmacologic effect. The relationship of cytosine arabinoside[3] (ara-C) sensitivity to kinase and deaminase levels in various tissues is probably the most significant work, but complete quantitation is not avail-

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[3] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

TABLE 1.—Illustrative blood flow rates (ref 6)

Species	Organ	Q/V (min^{-1})
Rat	Spleen	0.8
	Small intestine	0.9
	Bone marrow	0.29
	Walker 256 carcinosarcoma, transplanted	0.07
Rabbit	VX-2 carcinoma, transplanted	0.5
Dog	Spontaneous lymphosarcoma	1.2–18.0
Man	Adrenals	5.0
	Gray matter	0.8
	Liver + portal system	0.4
	Red marrow	0.09
	Muscle	0.01–0.05

able (1). With soluble enzyme systems such in vivo quantitation may be possible once the K_m and V_{max} of the purified enzyme have been established in vitro. Initial excursions into such studies with ara-C deaminase and kinase suggest the approach may be fruitful, but much more extensive experimentation is needed (2,3). The kinetics of non-soluble enzyme systems in vivo have yet to be modeled from in vitro measurements. New technologic advances will have to be made before this problem can be attacked.

Other less formidable problems for which data are available or can be collected involve blood flow, mixing volumes, and membrane permeability. With this type of information, pharmacokinetic models can give reasonable predictions of drug concentrations and residence times at various sites. Figure 1 illustrates a compartment of animal organ and indicates the minimal amount of information needed for predictive purposes. The volume of various compartments (V), the blood flow rate into the compartment (Q), and the membrane transport rate of drug (jA) are essential. Even with this information, predictions for active-site drug concentration can be grossly in error because of factors such as metabolism, binding, and intracellular compartmentalization whose relative importance can only be determined by experimentation. Figure 2 illustrates schematically the influence that some of the above basic parameters can have on drug concentration with time. The residence time of a drug is essentially prolonged with relatively smaller blood flow (Q/V) following a simple iv injection. Similar qualitative patterns are obtained by relative changes in

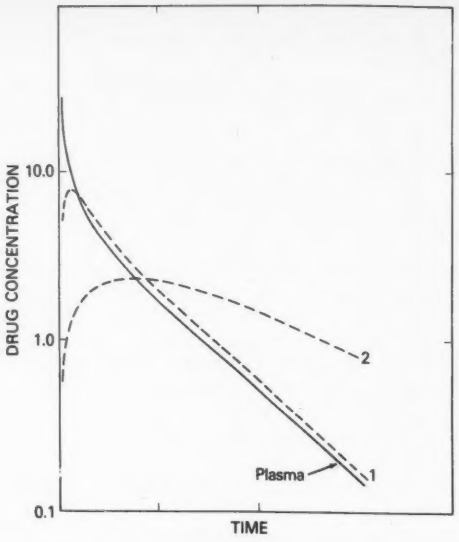

FIGURE 2.—Schematic diagram illustrating the effect of blood flow or membrane permeability on tissue-drug concentration relative to plasma-drug concentration.

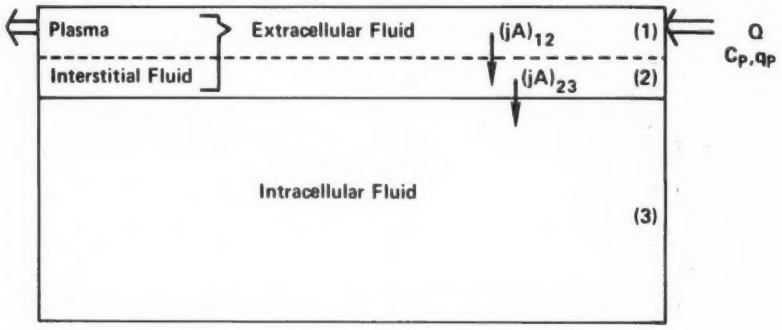

FIGURE 1.—Compartment showing inter- and intra-tissue transport (6). C_p and q_p are free and total drug concentrations in the plasma.

TABLE 2.—Membrane transport of methotrexate (ref 6)

Species	Cell or tissue	PA/V (min^{-1})
Mouse	L1210 leukemia	0.14–0.3
	Sarcoma 180	0.005
	Skeletal muscle	0
Rat	Small intestine	0.031
	Spleen	0.0082
	Bone marrow	0.013
Man	Granulocyte	0.01
	Small lymphocyte	0.003
	Lymphoblast (acute lymphocytic leukemia)	0.006–0.015

permeability. High flow or permeability relative to volume results in pattern 1 whereas relatively lower flow or permeability results in pattern 2 (fig 2). Such factors then can determine whether a drug is present at the active site in high concentration for a short period of time or a lower concentration for a longer period of time following a single injection into an animal. Table 1 illustrates the wide range of blood flows relative to tissue volumes in a variety of organs and tissues. The first impression created by these data is that blood flow to some organs may play a role in limiting drug delivery to the active site. However, the additional factor of membrane permeability must be considered together with blood flow and organ volume to put these factors in proper perspective. Only when flow relative to volume (Q/V) for a tissue is much less than the membrane permeability relative to volume (PA/V) is flow a limiting factor. Table 2 illustrates the wide range of membrane permeabilities to methotrexate[1] in a variety of tissues. Comparison of tables 1 and 2 shows that Q/V > PA/V in all organs where PA/V and Q/V are both documented, which indicates that transport limitation at the cell membrane is the limiting step with methotrexate, and this can range widely. Additional studies indicated that two organs, liver and kidney, behave pharmacokinetically as flow-limited systems, implying indirectly a relatively high permeability to methotrexate (4).

The central nervous system (CNS) is more complicated. The capillaries in the brain possess tight intercellular junctions, and this creates a blood-brain barrier to many drugs (5). Use of lipophilic drugs and intrathecal infusions of hydrophilic drugs are two methods used in attempts to circumvent this barrier problem. Pharmacokinetic modeling of antimetabolite drugs in the CNS has been limited. Quantitative data concerning cerebrospinal fluid (CSF) flow and capillary and cellular permeability are worthy of further study. Since a more detailed presentation of accomplishments to date is presented elsewhere in this Workshop, these will not be reviewed here. However, I would like to discuss a modeling attempt with methotrexate in the CSF of man because it illustrates one important point with practical clinical implications (6). Systemic pharmacokinetic behavior of the drug was coupled with CSF transport which included both diffusion and bulk flow. The model, confirmed with collected data, illustrates that methotrexate passes from the CSF to the blood and that the CSF serves as a reservoir to release drug slowly into the blood. The result is a much longer plasma-methotrexate concentration $> 2 \times 10^{-8}$ M from an intrathecal dose than from the same dose given iv with potential systemic toxicity to bone marrow and gastrointestinal mucosa. Here the opportunity presents itself to use citrovorum factor[5] iv to reduce systemic toxicity. Since iv citrovorum factor would be expected to penetrate the brain poorly, little reduction in methotrexate effect in the CNS would be anticipated.

Transport Competition and Citrovorum Rescue

The need to understand the relationship between concentrations of methotrexate and citrovorum factor was first elucidated with in vivo studies by Goldin et al (7). More recent studies in select in vitro systems indicate that citrovorum factor and methotrexate share a common transport system in a competitive manner. This suggests that at high blood-methotrexate concentrations higher citrovorum factor concentrations are required to prevent methotrexate toxicity than at lower methotrexate concentrations. Clinical data presented by Tattersall et al (8) indicate that this is indeed the case. These data indicated that marrow toxicity was manifested by all patients whose plasma-methotrexate concentration was above the estimated plasma-citrovorum factor concentration 48 hours after starting methotrexate treatment. Unpublished data in our laboratory suggest that in mice equal or higher concentrations of citrovorum factor are necessary to effect rescue to specific organs if the two drugs are administered simultaneously. Each tissue exhibits a characteristic ratio (methotrexate/citrovorum factor) varying from 1:1 to 1:10 for rescue. If, however, the methotrexate concentration in the blood is lowered by stopping a constant infusion or by normal clearance processes following a single pulse injection to below toxic levels (5×10^{-9} M in mice), then a minimal plasma concentration of citrovorum fac-

[1]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[5]Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

tor ($\sim 1 \times 10^{-8}$ M in mice) appears to be sufficient to hasten the recovery process from DNA synthesis inhibition so that lethal toxicity is prevented. For example, in the small intestine, deoxyuridine incorporation into DNA returns to control levels within 24 hours after stopping methotrexate infusion if citrovorum factor is present at the time the methotrexate infusion is stopped. However, if citrovorum factor is absent the recovery is only to 10% of control at 24 hours. This suggests to us that the rate-limiting steps for recovery from methotrexate toxicity lie in the production of the folate coenzymes.

Binding Factors

Drug can bind to blood proteins, extracellular proteins, cell membranes, or macromolecules within the cell. Methotrexate is a good example of the variety of binding exhibited by a drug and the consequences of such binding. Binding to blood proteins removes a portion of the driving force for transport and reduces clearances of the ultrafiltration type. This factor should be considered when attempting to correlate and relate in vitro with in vivo results.

Binding of methotrexate to the cell membrane has been suggested indirectly by pharmacokinetic modeling of spontaneous lymphosarcoma in dogs (9). Such membrane binding could play a role in interpreting location of the drug relative to its active site. The above pharmacokinetic analysis indicated the interplay of blood flow, mixing volumes, permeability, enzyme, and membrane binding in determining the intracellular concentration of free drug.

Binding of methotrexate to the enzyme dihydrofolate reductase has been characterized as stoichiometric (10). This means the dissociation constant of the drug-enzyme complex is around 10^{-10} M or less and suggests that little free drug exists in the presence of significant free enzyme. Such concepts have been derived from in vitro studies with purified enzyme and may not apply directly to enzyme in sites under different conditions of pH and protein interactions (11). Recently Goldman (12) has suggested the presence of a low-affinity intracellular binding site for methotrexate which controls tetrahydrofolate synthesis. In earlier studies Hakala (13) suggested a dissociation of the methotrexate-enzyme complex in Sarcoma 180 cells in vitro to explain methotrexate resistance. Mishra et al (14) reported data which estimated dihydrofolate reductase activity in vivo in the liver and small intestine of mice. These data suggested either a rapid dissociation of inhibitor-enzyme complex or a rapid synthesis of new enzyme. Enzyme activity in both

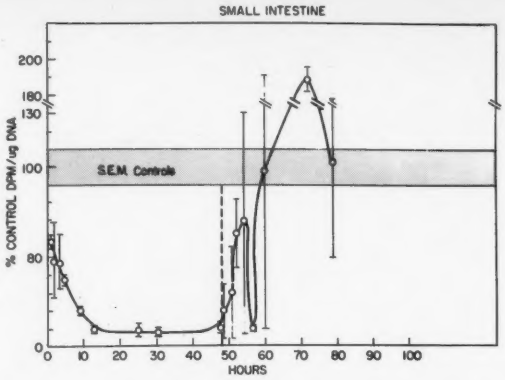

FIGURE 3.—Time course of incorporation of ^{3}H-6-deoxyuridine into DNA after sc infusion of methotrexate at 0.8 μg/hr for 48 hrs.

organs returned to at least 50% of control activity within 5 hours after a single methotrexate dose of 15 mg/kg in mice. We have suggested in a recent publication that binding to the enzyme, dissociation from the enzyme, and new enzyme synthesis are probably all involved in determining the degree of drug effect under conditions of constant drug exposure in vivo (15).

Kinetics of Biochemical Interactions

With in vivo experimental systems one is dealing with dynamically changing conditions rather than the constantly controlled conditions which are strived for in cell-free systems and partially exist in cellular systems in vitro. Administering a single dose of methotrexate to an animal and then following the pharmacologic effect in different tissues provide certain insights that cannot be achieved by studying pharmacokinetics alone in the whole animal or effects alone in vitro. Such studies with methotrexate have been reported by a variety of laboratories (14–16). Results of these studies suggest that different normal dividing tissues have different permeabilities and respond uniquely to similar extracellular fluid concentrations. As illustrated in figure 3, deoxyuridine incorporation into DNA of the small intestine is gradually inhibited and remains inhibited during a constant infusion of methotrexate which achieves a plasma concentration of 2×10^{-8} M. Recovery is rapid when the infusion is stopped if a critical time of about 48 hours is not exceeded. Bone marrow tissue is less permeable; therefore, it is inhibited less and is capable of recovery during infusion (15). A plausible explanation for these observations is that the rate of drug penetra-

tion to the enzymatic site cannot keep up with new enzyme synthesis.

A recent study by Sirotnak et al (18) comparing aminopterin[6] with methotrexate indicates that the probable reason for aminopterin's greater toxicity is a combination of its higher rate of influx and its lower rate of efflux in mouse small intestine. The end result is a relatively greater persistence of intracellular free aminopterin than methotrexate at equivalent initial doses. Whether this persistence of free drug results in greater toxicity because free drug is necessary to prevent dissociation of the drug-enzyme complex or because newly synthesized enzyme needs free drug to be inactivated requires further study to answer.

Limitations of Antifolates

Werkheiser (10) listed six factors that must be considered in order to evaluate the potential effects of a folate antagonist upon a tissue. In the intervening 10 years all of these factors have been actively studied: (*a*) time course of blood concentration; (*b*) cell permeability; (*c*) folic reductase level; (*d*) coenzyme level; (*e*) rate of DNA synthesis; and (*f*) growth fraction. New information has been gathered of which much has already been applied to practice clinically. These same factors no doubt must be thoroughly understood in order to achieve more successful treatment of CNS tumors with antimetabolites. One additional factor which has recently been explored in vivo is the selectivity of drug action as a result of specific salvage pathway utilization (19). Many previous in vitro studies have suggested that certain tumor lines utilize salvage pathways for DNA synthesis (20–22). However, little has been done with respect to in vivo exploitation of these suggestions. There is a paucity of information regarding distribution kinetics of the nucleosides and behavior of normal tissues in this regard. Recent data of Tattersall et al (23) have indicated that differences in response to methotrexate of five different mammalian cell lines in vitro could be attributed to variability in salvage pathway utilization. In vivo exploitation of such differences has not been attempted to any degree, but it may provide an opportunity to increase the selectivity of antimetabolites. The CNS may offer the best opportunity to explore this aspect because of its unique characteristics with respect to its discrete location anatomically and its biochemical and cellular kinetic characteristics. The limitations that apply to antimetabolite use in vivo, however, must be kept in mind and scrutinized carefully to determine whether they also apply to the use of normal metabolic substances used in the salvage mode in vivo. Concepts of blood concentration time course, permeability, binding of the salvage metabolites, and cell kinetics should be considered in the planning of such in vivo studies.

[6]Aminopterin: NSC-739; CAS reg. No. 54-62-6; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl]methyl]amino)benzoyl]-.

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Theoretic Limitations of Intracarotid Infusions in Brain Tumor Chemotherapy

Joseph D. Fenstermacher[1,*] and Alan L. Cowles[1,2,3]

SUMMARY

The drug distribution differences between arterial and venous infusion were studied by means of a computer model. The following transport variables were systematically altered to illustrate their individual actions on the flow of drug throughout the body: the site of drug administration, the rate of drug infusion, the rate of tissue perfusion or blood flow, the rate of metabolism and/or excretion (these two processes lead to a loss of drug and thus may be treated together), the rate of blood-tissue exchange (extraction fraction), and the tissue's affinity for the drug (partition coefficient). The tumor was considered to be located in the brain, the principal site of systemic toxicity was assumed to be the bone marrow, and the carotid artery was chosen as the site for arterial infusion. Curves showing the drug concentrations within the various tissue and fluid compartments over time were generated, first for the intracarotid and subsequently for the iv infusion sites. The results of such a set of computer runs were compared and the advantages (increased drug delivery to the brain and decreased drug uptake by the marrow) of the intracarotid site over the iv site were determined. Rapid carotid infusion of drug yielded very high peak concentrations in the brain. At low rates of tissue perfusion, the intracarotid infusion produced both a large peak concentration and a large exposure (concentration-time product) of drug to the tissue. With a carotid infusion, markedly diminished delivery of drug to the marrow was observed when the rate of metabolism-excretion was increased. Increasing or decreasing the rate of blood-brain exchange and the brain-blood partition of drug caused changes in the tissue uptake which were similar for both iv and intracarotid infusions, indicating that these transport properties have little importance in the choice of the drug infusion site.

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The distribution of drugs from the administration site to both normal and malignant cells is a complex function of a series of pharmacologic and physiologic factors, such as the route and schedule of the infusion and the rate of blood flow to the various organs and tissues (1). If the drug is to be useful in the treatment of brain tumors or any other disease, these factors must combine to yield an effective delivery of drug to the target or diseased cells without providing a markedly toxic amount of drug to normal cells. As a means of viewing the interplay of the factors which affect drug distribution, a computer model of blood-tissue transport for the entire body has been developed (2) and a number of drug infusion situations have been examined. For each specific combination of factors, a comparison in the drug uptake patterns between intra-arterial and iv infusion sites will be made.

Since the purpose of this study is to provide suggestions and guidelines for the chemotherapy of central nervous system malignancies, the tumor will be considered to be located in the left cerebral

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hemisphere, and thus the site for intra-arterial drug infusion will be the left carotid artery. The principal site of systemic toxicity will be assumed to be the marrow. Those physiologic and pharmacologic conditions which yield either higher brain uptake or lower marrow delivery of drug with the intracarotid route of administration will be indicated.

METHODS

The predictions of drug distribution were based on a model which has been previously described and used by Cowles and Fenstermacher (2). In brief, the model consisted of a number of blood and tissue compartments. The blood compartments which were used include the following: a central arterial compartment, the carotid artery, a central venous compartment, and the capillary compartments for each solid tissue. The tissue compartments were made up of single organs or groups of organs which have similar rates of blood perfusion and transport properties. They are: (1) lung tissue; (2) adrenals, thyroid, and kidney; (3) heart; (4) brain; (5) red marrow, lymphoid, and splanchnic tissues; (6) gonads and spinal cord; (7) skin, subcutaneous tissue, and skeletal muscle; and (8) yellow marrow and adipose tissue. Each compartment was appropriately scaled for size.

In setting up the model and making calculations for the uptake and distribution of the hypothetic drug, a number of assumptions were made. The more important of these are as follows: (*a*) the rate at which drug moves between blood and tissue is directly proportional to the activity gradient (no saturable transport processes); (*b*) each portion of the model consists of a single, well-mixed compartment in which the drug distributes uniformly; and (*c*) the rates of drug metabolism and excretion in a tissue are directly proportional to the activity of drug in that tissue.

The actual calculations were made by dividing the modeling period into a series of consecutive small time intervals (0.05 minute) during which the concentrations in the various compartments were considered to be constant. Based on the transport and metabolism parameters of each tissue and blood compartment, the amounts of drug passing between communicating compartments were calculated for each time period and new tissue concentrations of drug were determined. This cycle of calculations was repeated until only 2% of the infused drug remained in the body.

The variables in the model were the site of infusion (central vein or left carotid artery), the rate of infusion, the individual tissue:blood partition coefficients, the rate of blood-to-tissue exchange (the equilibrium or extraction fraction), the rate of drug metabolism, and the rate of tissue perfusion. Unless otherwise stated, the following set of values was used for the above variables: (1) a total drug administration of 100 mg, (2) an infusion rate of 25 mg/minute for 4 minutes, (3) a tissue:blood partition coefficient of 1.00, (4) a capillary extraction fraction of 0.8, and (5) a metabolic half-life of 30 minutes.

Since a computer model such as this provides a tremendous amount of data, only a small proportion of the total output can be presented. The concentrations of drug at the following sites were deemed of interest for the brain tumor chemotherapist: (*a*) the left carotid artery (the concentration profiles in the capillaries of the brain's left hemisphere would be similar to this); (*b*) the left brain (the assumed location of the tumor and subsequently referred to in the text as the *brain*); (*c*) the central arterial compartment; and (*d*) the red marrow and the lymphoid and splanchnic tissue (the presumed sites of drug toxicity and subsequently referred to in the text as the *marrow*). Graphs of the drug concentration over time plus two different quantities, the maximum or *peak* concentration and the concentration-time product or *exposure*, associated with the curves will be presented. In all cases data obtained from intracarotid and iv infusions under the same conditions will be presented together.

RESULTS AND DISCUSSION

Arterial Concentration-Time Profiles

In the presentation of data the arterial concentration profiles, which are virtually identical with their related capillary profiles, will be emphasized. This is done for two reasons. First, as shown by Eckman et al (3), the advantage of increased drug delivery to a tumor site or decreased drug uptake by susceptible normal tissues which an arterial infusion gives over a venous infusion is determined by the time course of the drug's concentration within the arteries which serve these sites and tissues. The concentration of drug in the artery and, subsequently, in its associated capillaries is the driving force for the tissue uptake process.

Second, the transport and metabolic properties of most types of brain tumors are unknown; therefore, attempts to choose precise values for these variables with a particular drug and tumor are not possible. Yet, as just indicated, these factors are relatively unimportant in determining the delivery advantage of an arterial infusion since the tissue's transport and metabolic systems only work upon the amount of drug which is brought to them by the circulatory system and function in an identical fash-

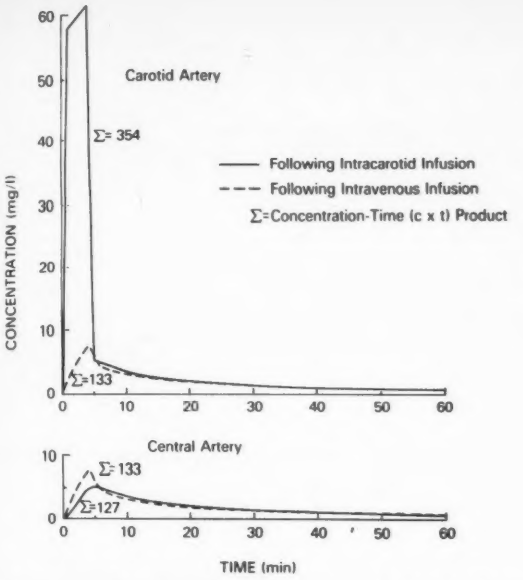
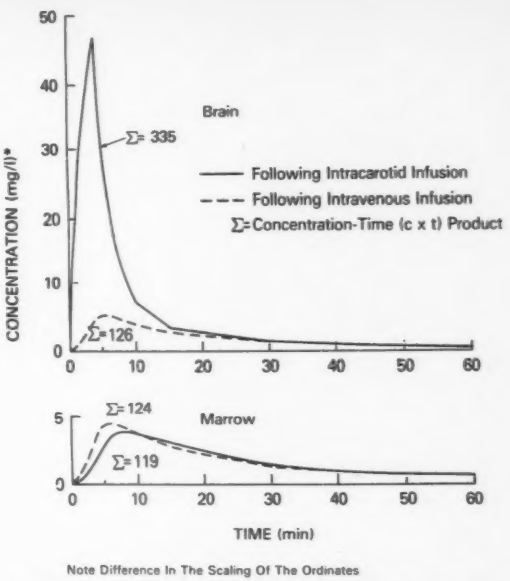


FIGURE 1.—Computer-generated time courses of model drug concentrations in carotid and central arteries for both intracarotid and iv infusions of 25 mg/min for 4 mins. The basic physiologic and pharmacologic conditions, as described in the Methods section, were used to produce these data.



Note Difference In The Scaling Of The Ordinates

FIGURE 2.—Computer-generated time courses of model drug concentrations in brain and marrow tissues for both intracarotid and iv infusions of 25 mg/min for 4 mins. The basic physiologic and pharmacologic conditions, as described in the Methods section, were used to produce these data.

ion regardless of the site, arterial or venous, of drug infusion. Therefore, the most reliable information for evaluating the drug distribution differences in these two routes of administration is provided by the time course of drug concentration in the pertinent arteries of the body.

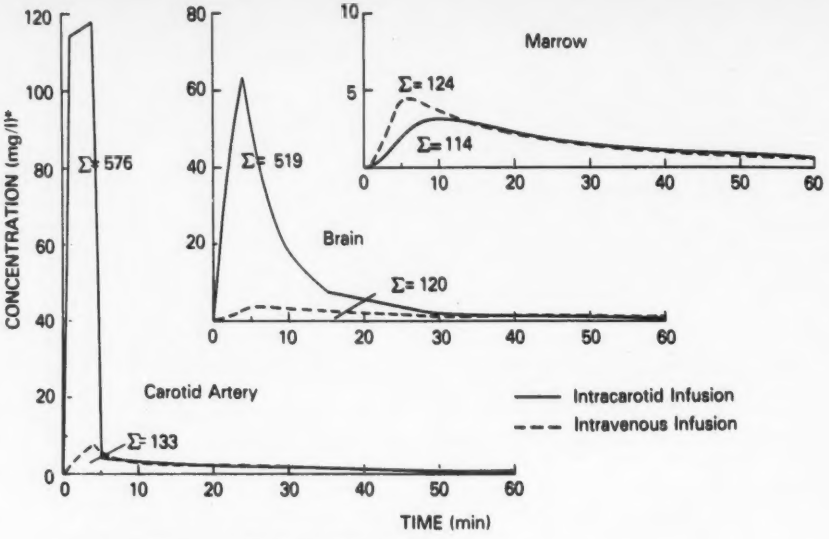
Basic Conditions

Figure 1 shows the concentration of drug in the carotid and central arterial compartments over time with both routes of administration under the previously defined basic conditions; figure 2 gives the companion data for the brain and marrow. As can be seen from these graphs, the arterial infusion leads to higher peaks and exposures for the carotid artery and brain tissue and lower peaks and exposures for the central arterial and marrow compartments than does the venous infusion. Such a qualitative difference between these two routes of drug administration probably always exists; however, the magnitude of the differences can be much smaller or larger than shown in figures 1 and 2.

As would be expected, the peaks and exposures obtained in the carotid and central arterial compartments for the iv infusion were identical (7.5 mg/liter and 133 mg/minute/liter, respectively). This similarity was found in all cases. Furthermore, the drug concentration data in the central arterial compartments for the intracarotid and iv infusions were always fairly similar, although the values of the peaks and exposures for the intracarotid infusion were consistently lower than those for the iv infusion. Because of these similarities, no further graphs of the central arterial data will be presented.

Infusion Rate

The effects of intravascular infusion rate upon the blood and tissue concentration peaks and exposures are presented in table 1 (the middle row of data gives the values for the basic conditions). The rate of infusion very strongly affects the peak concentration but has no effect upon the exposures. Hence, if the achievement of a very high but very brief concentration of drug in the brain is advantageous, a rapid arterial infusion would be effective. If the drug's effectiveness is directly related to tissue exposure, then the rate of drug administration is quite unimportant.



* Note Differences In The Scaling Of The Ordinates

FIGURE 3.—Computer-generated concentration-time profiles of model drug in the carotid artery, brain, and marrow for both intracarotid and iv infusions of drug when brain blood flow has been reduced to 50% of normal.

Blood Flow

Figure 3 shows the effects of decreasing tissue perfusion on the carotid artery, brain, and marrow concentration curves. When the carotid artery's rate of blood flow is 50% that of the basic situation, the carotid artery and brain tissue peaks and exposures of drug following intracarotid infusion were markedly elevated. Furthermore, the marrow's peak (3.1 mg/liter) and exposure (114 mg/minute/liter) for the intracarotid infusion were slightly lowered relative to the basic case (3.7 mg/liter and 119 mg/minute/liter, respectively). On the other hand, the blood and tissue concentration profiles with the iv infusion of the drug were quite similar regardless of the brain's perfusion rate.

When the rate of blood flow was increased, the opposite effects were observed; therefore, in the situation where the brain tumor's rate of perfusion is lower than that of normal brain tissue, an intracarotid infusion seems to offer advantages (higher brain peaks and exposures and slightly lower concentration values for marrow) over an iv infusion.

Metabolic Rate

Changing the rate of metabolism-excretion had very little effect on the carotid and central artery

TABLE 1.—Effects of infusion rate upon blood and tissue peak concentrations and exposures

Infusion schedule (mg/min)	Infusion site*	Peak concentration (mg/liter)				Exposure (mg/min/liter)			
		Carotid artery	Brain tissue	Central artery	Bone marrow	Carotid artery	Brain tissue	Central artery	Bone marrow
100 × 1 min	ic	230	77	5.5	3.8	354	335	127	119
	iv	12	5.4	12	4.6	133	126	133	124
25 × 4 mins	ic	62	47	5.2	3.7	354	335	127	119
	iv	7.5	5.0	7.5	4.4	133	126	133	124
1 × 100 mins	ic	3.5	3.3	1.2	1.1	354	335	127	119
	iv	1.2	1.2	1.2	1.1	133	126	133	124

*ic = intracarotid.

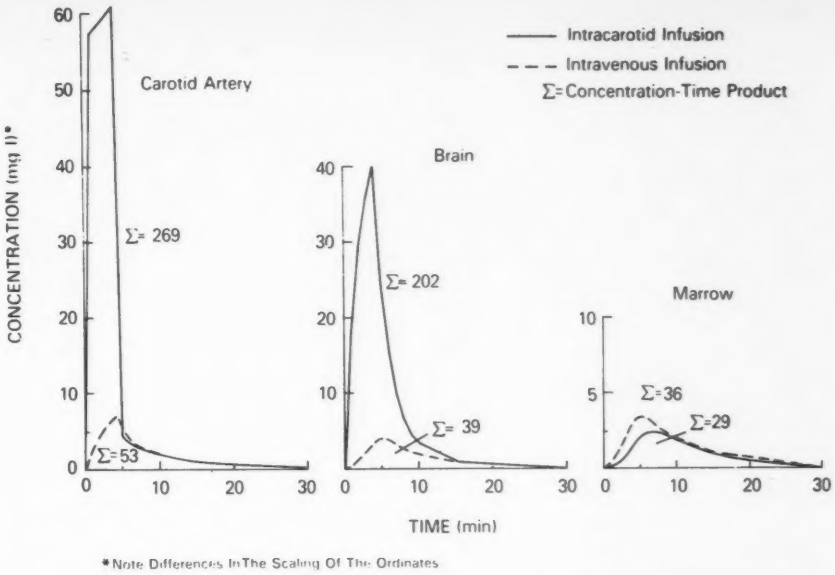


FIGURE 4.—Computer-generated concentration-time profiles of model drug in the carotid artery, brain, and marrow for both intracarotid and iv infusions of drug when the rate of metabolism is high (half-life = 5 mins).

peaks regardless of the route of drug administration. As a result, the brain and marrow peaks were relatively unaffected by changes in metabolism and excretion. In contrast, the exposures were markedly dependent on the rate of metabolism. Figure 4 illustrates a case of rapid metabolism-excretion (half-life = 5 minutes). The exposures of the carotid artery and brain following intracarotid administration of the drug are 75% and 60% of their basic values, respectively, whereas the marrow exposure was a mere 24% of its basic value. The exposures for arterial blood and the tissues following iv infusions were also quite low.

For a slow rate of drug metabolism-excretion the opposite exposure results were obtained. The blood and tissue exposures for both the intracarotid and the iv cases increased. For example, the marrow exposures in the basic case (half-life = 30 minutes) were 119 (intracarotid) and 124 (iv) mg/minute/liter, whereas in the situation of slow drug metabolism-excretion, these values were 348 and 352 mg/minute/liter, respectively.

From the preceding results, an intracarotid infusion seems to offer a chemotherapeutic advantage when the rate of drug metabolism-excretion is rapid because of the diminished delivery of drug to the presumed sites of toxicity rather than an increased uptake of drug by the malignant cells within the brain. A low rate of metabolism-excretion clearly reduces the drug distribution superiority of intracarotid infusions.

Tissue Factors

Two tissue transport factors, the partition coefficient (λ), which is a measure of the tissue's drug uptake capacity, and the extraction fraction (F), which is a measure of the drug's rate of blood-brain exchange, have independent effects on both the arterial and the tissue time courses of drug concentration. Two extreme conditions, which illustrate most clearly the separate effects of changing λ or F upon the concentration profiles, will be considered.

The results of varying either the λ or the F upon the arterial concentration of drug are most evident when the λ or F of the drug is increased or decreased simultaneously for every tissue of the body. On the other hand, altering the drug's F or λ for one tissue alone has minimal effects on the blood's concentration-time course but has prominent effects upon that particular tissue's concentration profile. Thus, the distribution data for these two extreme conditions, changing the drug's λ or F for either all of the tissue compartments or only a single compartment, were generated.

Varying All Tissue Factors

Simultaneously increasing the tissue:blood λ for all organ compartments lowers the concentration peaks and exposures of both the carotid and central artery regardless of the site of intravascular drug administration. For example, raising the λ of all compartments from 1.0 (basic condition) to 4.00 decreased the carotid artery peak from 61.5 to 59 mg/liter and its exposure from 354 to 281 mg/minute/liter for an intracarotid administration of drug. A similar decrease (about 70 mg/minute/liter) in the carotid artery's exposure with iv infusion and in the central artery's exposure with both routes of drug infusion was observed. As would be expected from the preceding results, decreasing the drug's tissue:blood λ increased the peaks and exposures of both the carotid and central arteries.

A parallel set of arterial concentration effects is caused by simultaneously varying the blood:tissue F for all organ compartments. As the drug's F is decreased, the arterial peaks and exposures are increased. Lowering F from 0.8 (basic condition) to 0.1 increased the arterial peaks by 6–7 mg/liter and exposures by about 330 mg/minute/liter for both routes of intravascular drug administration. Concentration effects which were fairly similar in magnitude but opposite in direction were seen when the drug's blood:tissue F was increased.

Blood-Brain Exchange

As mentioned in the section on Tissue Factors, varying the drug's rate of blood-brain exchange (performed in our computer modeling by altering the brain F) had little or no effect upon either the carotid or central artery's concentration-time course; furthermore, since the marrow F was not changed, the drug's concentration profile in this tissue compartment would be unchanged. Therefore, the arterial and marrow data presented in figures 1 and 2 for the basic conditions are also representative for both arteries and the marrow with intracarotid and iv infusion cases where only the brain's F is varied.

Since the carotid artery's concentration profiles of drug are virtually unaffected by the F of the brain, any changes in the brain's uptake and loss of drug must be caused mainly by changes in the rate of blood-brain exchange. Figure 5 shows that the brain peaks and exposures were increased as F was enlarged from 0.1 to 1.0. Over the range of Fs, the brain tissue's drug exposure increased by 37% for both intracarotid (339/247) and iv (127/93) infusion cases. In addition, the intracarotid:iv exposure ratios for F = 0.1 (247/93) and F = 1.0 (339/127) were

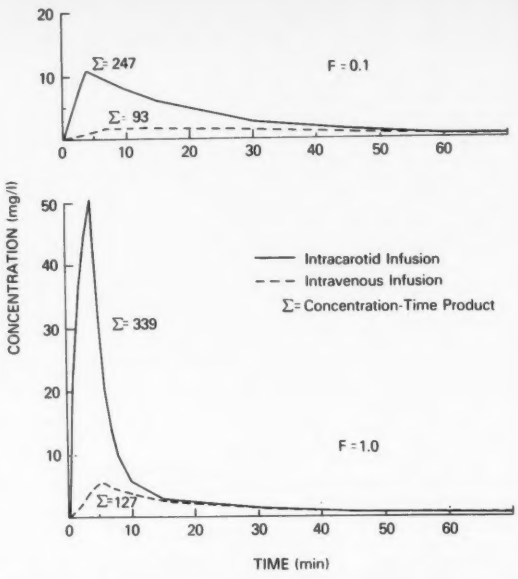


FIGURE 5.—Computer-generated time courses of model drug concentrations in brain tissue for both intracarotid and iv infusions of drug at 2 different blood-brain extractions or Fs, 0.1 and 1.0.

identical (both equal to 2.66), thus indicating no proportional increase in drug exposure between intracarotid and iv infusions for more rapidly exchanging compounds. Therefore, an intracarotid infusion of a drug which has a high brain capillary F provides some advantage in terms of the brain peak concentration but no particular gain in terms of brain exposure.

Brain λ

In general, changes in the brain:blood λ from 0.25 to 4.0 had only small effects upon the drug concentration profiles of the carotid and central arteries. As indicated in figure 6, large alterations in the brain tissue peaks and exposures were generated by varying the drug's brain λ. The brain peaks ranged from 15 (λ = 0.25) to 76.6 (λ = 4.0) mg/liter for the intracarotid infusion cases and 1.8 (λ = 0.25) to 9.3 (λ = 4.0) mg/liter for the iv infusion cases. In connection with this, the data demonstrated that the marrow peaks following iv infusion of the drug remained relatively constant as the brain λs were changed, but that the marrow peaks following intracarotid drug administration decreased from 4.5 to 2.2 mg/liter as the brain λs were increased from 0.25 to 4.0. These intracarotid versus iv infusion differ-

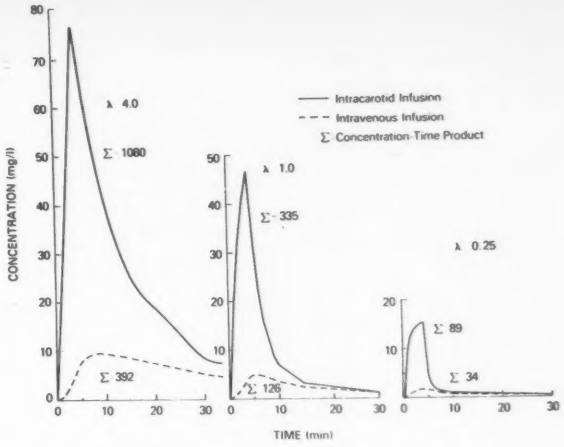


FIGURE 6.—Computer-generated concentration-time profiles of drug in brain for both intracarotid and iv infusions of drug using 3 different values for the brain:blood λ, 4.0, 1.0, and 0.25.

ences in the marrow peaks were a reflection of small but significant concentration variations for the central artery.

As the brain λ changed from 0.25 to 4.0, the brain tissue exposures for both the intracarotid and iv infusions increased more than tenfold; however, the intracarotid:iv drug exposure ratios were quite similar for each of the brain λs which was modeled. For example, at λ = 0.25, the intracarotid:iv exposure ratio for the brain was 2.62 (89/34) and at λ = 4.0, the ratio was 2.76 (1080/392). This slight increase in the intracarotid:iv ratio of brain was caused by the small change in the carotid artery's drug exposures which happen when the brain λ goes from 0.25 (carotid artery ratio = 2.62) to 4.0 (carotid artery ratio = 2.75).

Some diminution (127–95 mg/minute/liter) in the marrow exposure with an intracarotid infusion occurred as the brain λ increased, whereas, with the same set of conditions, a much smaller (11%) decrease in the marrow exposure was found with an iv infusion.

The intracarotid administration of a drug with a high affinity for the brain is superior if a high peak concentration is desirable; however, if the tissue exposure is the important drug uptake parameter, then an intracarotid infusion offers only a slight advantage.

COMMENT

Both the exposure and the peak concentration were obtained and presented from these computer-generated data. As discussed by Mellett in a recent review (4), the exposure seems to be the more important of these two parameters in predicting drug effectiveness.

Examination of the various arterial drug concentration profiles (figs 1, 3, and 4) indicates that the divergences between the intracarotid and iv curves exist mostly for the duration of the infusion. A similar perusal of the brain and marrow drug data (figs 2–6) shows that the intracarotid and iv infusion differences in these two tissues persist for a somewhat longer period of time (15–30 minutes with the conditions modeled in this study). As previously emphasized by Eckman et al (3), the delivery advantage of an intra-arterial infusion occurs only during the first passage (and subsequent tissue exchange) of the drug through the infused artery's capillary system. The intra-arterial drug distribution superiority which is established during the administration period is quickly dissipated when the infusion is stopped unless the drug is tightly bound within the tissue.

The computer data also suggested that the advantage of an intracarotid infusion would be increased if the rate of blood flow through this vessel and, consequently, through the microvessels which it supplies within the tumor, was low. Since the choice of major accessible cerebral arteries is limited, the chemotherapist must choose the artery for drug administration on the basis of the tissue site or sites which it serves. Since this constraint usually restricts the choice of vessels to one or the other of the carotid arteries, little or no opportunity to select a

low-flow vessel for intra-arterial administration of the drug exists. Lowering the rate of flow through an artery, such as the carotid, by mechanical or pharmacologic means is possible but clinically impractical.

Our computer studies indicate one situation: rapid drug metabolism and/or excretion, in which an intracarotid infusion seems to be superior to an iv infusion. Ideally, if there is a drug which is active against the cells of the tumor over a relatively short period of time and is completely or nearly completely cleared from the blood by metabolism or excretion during the drug's first passage through the lungs, that agent should be given by intracarotid infusion. This principle is applicable not only for brain tumor chemotherapy, but also for tumors at other sites within the body. The search for such a drug would seem to be worth a reasonable amount of time and effort.

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Physicochemical Considerations and Pharmacokinetic Behavior in Delivery of Drugs to the Central Nervous System [1]

L. B. Mellett [2,3,*]

SUMMARY

The blood-brain barrier is not as rigid or as formidable as once believed. When experimental tumors of the central nervous system (CNS) and tumors of non-CNS origin are implanted intracerebrally (ic), they will grow and kill the host. It was once thought that only a few select agents, such as the nitrosoureas, were capable of crossing the blood-brain barrier and inhibiting tumor growth. There are now considerable data available which indicate that a variety of agents such as cytosine arabinoside, methotrexate, 6-mercaptopurine, 5-fluorouracil, procarbazine, melphalan, and cyclophosphamide, as well as the nitrosoureas, may significantly influence the growth rate of experimentally implanted ic tumors. A number of physicochemical factors may influence the penetration of the blood-brain barrier. These include pKa values, log-P values, and molecular size. Brain-level measurements indicate that most drugs have some accessibility to the CNS. Maximal effects against sensitive, ic implanted tumors are attained through maximal scheduling. Effective drug combinations should be sought to further enhance antitumor effects in the CNS.

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Traditionally, chemotherapists have employed various animal tumor models to evaluate the responsiveness of the tumor to various anticancer drugs. Such tumor models have also been employed to evaluate the ability of the drugs to cross the blood-brain barrier. When experimental tumors of the central nervous system (CNS) and tumors of non-CNS origin are implanted intracerebrally (ic), they will grow and kill the host. Failure of ic implanted tumor to respond to parenterally administered drug was generally interpreted as poor penetration of the brain by the drug (1). The blood-brain barrier is not as rigid or formidable as once believed. It is now clear that a number of antimetabolites, alkylating agents, and related drugs have some access to the CNS and show marked activity against a number of different ic implanted rodent tumors.

I have chosen to limit my discussion to the L1210 murine leukemia model in mice. There are, however, several biologic considerations which should be examined with this model as well as any other model employed in the evaluation of the ability of anticancer drugs to cross the blood-brain barrier. These considerations may seem obvious, but they have sometimes been ignored in the past. The following is a summary of these considerations.

A.—Drug-responsive tumors should be selected as targets. The tumors should respond to a given agent after ip implantation. Failure of ic implanted tumor to respond will then indicate poor penetration of the brain. When the L1210 model is employed in the evaluation of methotrexate (MTX) against ic implanted tumor cells, an ambivalent (±) result is obtained. However, this may, in part, be explained by the limited response of ip L1210 to MTX. It is possible to demonstrate increased lifespan (ILS) with MTX versus ip L1210; however, "cures" are rarely, if ever, effected. My point here is that the L1210 model is not ideal for the evaluation of the

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[3] The technical assistance and literature search provided by Melissa Johnson are gratefully acknowledged.

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TABLE 1.—Schedule dependency of ara-C vs ic implanted L1210

L1210 cell implant size	Ara-C therapy mg/kg/dose	Ara-C therapy Schedule	% ILS	No. of 30–45-day survivors/ total	Tumor cells present at end of therapy: Dying animals	Tumor cells present at end of therapy: Survivors
10^5	35	qd, 1–15 days	157	0/10	1.0×10^9	—
10^4	35	qd, 1–15 days	162	0/10	3.2×10^8	—
10^3	35	qd, 1–15 days	155	0/10	5.7×10^7	—
10^5	15	q3h × 8, Days 2, 6, 10, and 14	275	1/10	ca 1	2
10^4	15	q3h × 8, Days 2, 6, 10, and 14	205	8/10	4	0.2
10^3	15	q3h × 8, Days 2, 6, 10, and 14	—	10/10	—	<0.1
10^5	7.5	q3h × 8, Days 2, 6, 10, and 14	187	1/10	1.8×10^9	2
10^4	7.5	q3h × 8, Days 2, 6, 10, and 14	200	0/9	10	—
10^3	7.5	q3h × 8, Days 2, 6, 10, and 14	200	4/10	ca 1	0.9

ability of MTX to penetrate the blood-brain barrier.

B.—Optimal schedules should be employed. The optimal schedule developed for the treatment of ip implanted tumor should be employed for ic implanted tumor. I will have more to say about this aspect.

C.—The size of the implant (ideally, about 1×10^5 L1210 cells) should be the same for ip and ic implantation studies. If the ic implant is too large, or if treatment is delayed, the response to a drug may be negative and the drug's ability to penetrate the CNS underestimated. Table 1 shows the response of ic implanted L1210 for several schedules with cytosine arabinoside (ara-C). These data are presented to demonstrate schedule dependency and the importance of selecting the optimal schedule of a drug for evaluation versus ic implanted L1210. The optimal schedule for treating ic implanted tumor is 15 mg/kg, q3h × 8, Days 2, 6, 10, and 14. This schedule is also optimal for the ip implanted tumor.

In addition to the biologic considerations, there are physicochemical and pharmacokinetic factors which should be employed in the evaluation of the ability of anticancer drugs to cross the blood-brain barrier.

A.—Log-P values are a measure of the partitioning of drug between octanol and water. High, positive log-P values (+1.0, +2.0, +3.0, indicating a ten-, 100-, and 1000-fold partitioning in favor of octanol) suggest a high lipid solubility and a high likelihood of good penetration into the CNS. Negative log-P values indicate greater solubility in water and a reduced capacity to penetrate the CNS.

B.—pKa values can provide an estimate of the degree of ionization at physiologic pH. The unionized form of the drug will more readily penetrate cellular barriers.

C.—Molecular size may be an important determinant of a drug's ability to penetrate the blood-brain barrier. A number of high molecular-weight agents (> 500 daltons) with high, positive log-P values and acceptable pKa values fail to penetrate the brain significantly.

D.—Pharmacokinetic factors such as blood flow, peak serum levels of drug, half-life of the drug, and tissue distribution play an important role in determining the optimal therapeutic schedule.

E.—I have not purposely ignored the role of protein binding of drugs, but from the analysis of the available data it was not possible to assign a definitive role for this phenomenon in the response of ic implanted L1210.

Physicochemical properties, pharmacokinetic behavior, and distribution of antimetabolites are considered in relation to the response of ic implanted L1210 in mice in table 2. In this table, and the following two tables, log-P values and pKa values were largely derived from the work of Hansch and his co-workers and from other literature sources (2–4).

Pharmacokinetic values for mice were derived from studies in our laboratories and from various literature sources. Peak serum and brain levels of drug were used to compute brain-serum ratios. The responses of ic implanted L1210 in mice after treatment with optimal therapeutic schedules for each agent were obtained through the courtesy of Dr. Frank Schabel, Jr, of this institute (published and unpublished results).

With the exception of 6-mercaptopurine (6-MP), all of the antimetabolites listed have negative log-P values, indicating a preferential partitioning into water and a reduced capacity to penetrate the blood-brain barrier.

pKa values indicate a great deal of variability in the percent ionization at pH 7.4. It ranges from < 1% for ara-C to > 99% for MTX (66% for 6-MP and 83% for 5-fluorouracil [5-FU]).

TABLE 2.—Physicochemical properties, pharmacokinetic behavior, and distribution of antimetabolites and response to ic implanted L1210 in mice

Agent*	Log-P value	pKa value	Dose (mg/kg) and route	Average serum half-life (mins)	Peak serum levels (μg/ml)/time (mins)	Peak brain levels (μg/g)/time (mins)	Ratio of brain:serum	Response of ic implanted L1210†
Ara-C‡	−0.79	4.5	50 ip	25	35/15	3.1/30	0.09	+
Ara-C‡	−0.79	4.5	50 ip	25	41/15	4.3/30	0.11	+
MTX (5)	−1.85	4.7	10 ip	25	14/5	1.5/10	0.11	±
6-MP (6,7)	−0.01	7.7	100 ip	50	76/10	13.4/10	0.19	+
6-TG (6)	−0.07	—	70 ip	25–60	44/5	7.0/10	0.16	−
5-FU§	−0.95	8.1	50 iv	15	39/15	4.2/15	0.10	−
5-FU§	−0.95	8.1	50 iv	15	6.9/60	2.3/60	0.33	+
5-FU (8)	−0.95	8.1	200 iv	15	0.32/120	0.18/120	0.59	+

*Nos. in parentheses represent ref Nos. for original pharmacokinetic and serum and brain level studies.
†− = ILS < 25%; + = ILS > 25%.
‡Mellett LB, and Mulligan LT. Unpublished data.
§Furner RL, and Mellett LB. Unpublished data.

Pharmacokinetic values indicate a short half-life in the mouse for the various drugs listed. Brain-serum ratios at or near peak serum and brain levels range from 0.09 to 0.59 and indicate a limited but positive penetration into the CNS.

The response (+ = > 25% ILS) of ic implanted L1210 was positive for all drugs except 6-thioguanine (6-TG) and was marginal for MTX. In the case of 6-TG, only a single schedule has been investigated. The marginal response to MTX may only reflect the general low responsiveness of L1210 to this agent.

Physicochemical properties, pharmacokinetic behavior, and distribution of antibiotics and plant products in relation to the response of ic implanted L1210 are shown in table 3.

Log-P values range from +1.27 to +3.21 for the various drugs listed, which indicates high lipid solubility. Serum half-lives are generally longer for this group of agents than for the antimetabolites. Brain-serum ratios, however, indicate poor penetration into the CNS. Both daunorubicin and actinomycin D were negative when tested against ic implanted L1210. The low brain-serum ratios for the other agents suggest that they, too, would likely produce negative results in this test. The failure to penetrate the blood-brain barrier is attributed to the large molecular size of these drugs.

Physicochemical properties, pharmacokinetic behavior, and distribution of alkylating agents and related drugs are considered in relation to the response of ic implanted L1210 in table 4.

Log-P values for alkylating agents and related compounds range from −0.24 to +3.30. All of the

TABLE 3.—Physicochemical properties, pharmacokinetic behavior, and distribution of antibiotics and plant products and response to ic implanted L1210 in mice

Agent*	Log-P value	pKa value	Dose (mg/kg) and route	Average serum half-life (mins)	Peak serum levels (μg/ml)/time (mins)	Peak brain levels (μg/g)/time (mins)	Ratio of brain:serum	Response of ic implanted L1210†
Daunorubicin (9,10)	+1.83	—	10 ip	30 for 1 hr 260 after 1 hr	1.5/5	<0.1/5	<0.06	—
Adriamycin (11)	+1.27	—	10 ip	10 for 1 hr 180 after 1 hr	10.5/0	<0.1/10	0–0.06	NT
Actinomycin D (12)‡	+3.21	—	0.6 ip	20 for 1 hr 120 after 1 hr	0.3/5	<0.03/10	<0.10	—
Bleomycin (13)	—	—	50 sc	ca 30	39.0/60	<1.5/60	<0.04	NT
Vincristine§ (parent drug)	+2.82	5.5	3.0 ip	68	0.8/30	0.03/30	0.04	NT

*See explanation in table 2.
†− = ILS < 25%; NT = not tested.
‡Galbraith WD, and Mellett LB. Unpublished data.
§El Dareer SM, and Mellett LB. Unpublished data.

TABLE 4.—Physicochemical properties, pharmacokinetic behavior, and distribution of alkylating agents and related drugs and response to ic implanted L1210 mice

Agent*	Log-P value	pKa value	Dose (mg/kg) and route	Average serum half-life (mins)	Peak serum levels (μg/ml)/time (mins)	Peak brain levels (μg/g)/time (mins)	Ratio of brain:serum	Response of ic implanted L1210†
Cyclophosphamide‡ (parent drug)	+0.63	None	100 ip	25	84.5/5	25.0/15	0.29	+
Cyclophosphamide‡ (metabolites)	—	—	100 ip	ca 25	75.0/10	8.1/10	0.11	+
Melphalan§	—	—	10 iv	41	4.3/5	0.87/5	0.20	+
BCNU (14,15)	+1.53	None	20 ip	ca 20	24.0/5	12.0/5	0.5–1.5	+
CCNU (16)	+2.83	None	—	ca 30	—	—	4.0–5.0 at 7 mins	+
Methyl-CCNU	+3.30	None	60 ip	ca 50	6.2/15	—	—	+
DTIC	−0.24	4.6	317 ip	ca 35	—	—	0.10	−
BIC (17)	+1.54	—	1300 ip	—	<0.1/2–24 hrs	<0.1/2–24 hrs	¶	−
Procarbazine**	+0.06	6.66	100 ip	20–30	42.0/5	—	0.8–1.2 (through 6 hrs)	+

*See table 2 for explanation.
†− = ILS < 25%; + = ILS > 25%.
‡Mellett LB. Unpublished data.
§Furner RL, and Mellett LB. Unpublished data.
¶Very low concentrations in brain.
**Oliverio VT. Pharmacologic disposition of procarbazine. *In* Proceedings of the Chemotherapy Conference on Procarbazine (Matulane: NSC-77213): Development and Application (Carter SK, ed). Bethesda, Md, National Cancer Institute, 1970, pp 19–28.

materials have relatively short half-lives in vivo. Brain-serum ratios range from 0.10 to 5.0, and the response to ic implanted L1210 was positive for all drugs except the imidazole carboxamides, DTIC and BIC.

It seems apparent that most drugs have some access to the CNS. In addition to the classic considerations of lipid solubility, degree of ionization, and protein binding, it seems that molecular size plays an important role in determining penetration of the blood-brain barrier.

A significant element missing in this evaluation is the minimum concentration of the various drugs, and the time necessary to maintain this concentration, to provide significant tumor cell kill. With the L1210 model we are often guided with respect to these factors by tissue culture studies. In the case of human disease, such guidance is not so readily available.

In terms of combination chemotherapy versus ic implanted L1210, the results presented suggest that antimetabolites combined with alkylating agents and related compounds could produce enhanced responses with sensitive tumors of the CNS.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
BIC: NSC-82196; CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6
DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-
6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-
Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride
6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

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Regulatory Control of Tetrahydrofolate Coenzymes in Folate Auxotrophs[1]

Alberta M. Albrecht[2,*]

SUMMARY

The interrelated enzymic reactions of folate metabolism are presented and key tetrahydrofolate-producing reactions are emphasized. As observed with the methotrexate (MTX)-resistant mutant strain *Streptococcus faecium* var. *durans*/A_R, the regulatory roles of serine and purines in controlling their own synthesis by the repression of enzymes required for co-factor synthesis are reviewed. Positive induction of the dihydrofolate reductase activity of this mutant by folate and the antagonism of the folate effect by purines and thymine are discussed. A protective agent of the reductase-active protein, MTX is viewed also as a "positive" inducer of dihydrofolate reductase. Preliminary studies with L1210 leukemia-bearing mice and the murine leukemia ERLD in vitro suggest that citrovorum factor (CF) also triggers a positive induction of the reductase of the small intestine and of ERLD cells without apparently influencing the reductase level of L1210 in vivo. The possibility that control mechanisms, by which MTX and CF indirectly regulate enzyme synthesis in drug-stressed, CF-rescued cells, contribute to the success of high-dose MTX-CF rescue therapy is introduced.

[Cancer Treat Rep 61:533–538, 1977]

The central biochemical theme of the greater portion of this Workshop is the inhibition of folate-dependent metabolism and/or the rescue of normal cells from the toxicity of high-dose antifolate therapy. For biochemical orientation, it is appropriate at this early stage to review and underscore several interrelated facts (1).

BIOCHEMICAL BACKGROUND

Because folate metabolism involves the mobilization of one-carbon groups in the synthesis of amino acids and precursors of nucleic acid through mediation by tetrahydrofolic acid (tetrahydrofolate), the enzymic synthesis of tetrahydrofolate commands interest. Today, as throughout the past 20 years, researchers and clinicians justifiably maintain an active interest in the mammalian dihydrofolate reductase which catalyzes the reduction of both folate and dihydrofolate. Of fundamental biochemical importance is the action of this enzyme in catalyzing the reduction of dietary folates and the regeneration of tetrahydrofolate intracellularly. Because it is sensitive to antifolates, this enzyme continues to be a prime target in chemotherapy. The related phenomena of elevated enzyme levels and altered sensitivity to methotrexate[3] (MTX) in MTX-resistant cells (1,2) have indeed amplified the attention given to this enzyme.

Vital to unrestricted folate-dependent metabolism, dihydrofolate reductase, however, is not the key which provides mammalian cells with their first pulse of tetrahydrofolate. The identification of 5-methyltetrahydrofolate as the major plasma folate (3) and the recognition of its apparently facile transport (4) have led to the assumption that the reaction by which the methylated folate provides cells with tetrahydrofolate is the intracellular key. This introductory reaction is catalyzed by methyltetrahydrofolate homocysteine cobalamin methyltransferase or methionine synthetase (fig 1, enzyme 1). By this

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[3]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

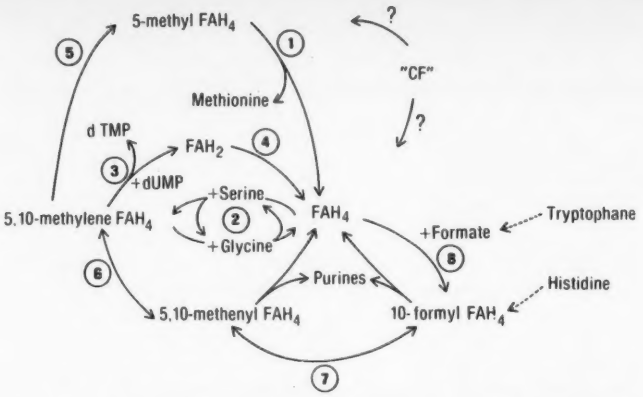

FIGURE 1.—Interrelated enzymic reactions of folate metabolism catalyzed by: (1) methionine synthetase; (2) serine hydroxymethyltransferase; (3) thymidylate synthetase; (4) dihydrofolate reductase; (5) methylenetetrahydrofolate reductase; (6) methylenetetrahydrofolate dehydrogenase; (7) methenyltetrahydrofolate cyclohydrolase; and (8) formyltetrahydrofolate synthetase. FAH_4 = tetrahydrofolate.

reaction, homocysteine is methylated (5–7) and tetrahydrofolate becomes available for the subsequent formation of its mono-carbon coenzyme derivatives (fig 1). Because the overall equilibrium of the reduction of 5,10-methylenetetrahydrofolate (enzyme 5) is far on the side of 5-methyltetrahydrofolate formation (8), there is essentially insignificant conversion of the 5-methyl- to the 5,10-methylene-derivative.

Serine, presumably, is the chief source of one-carbon units for the synthesis of these coenzymes in most mammalian cells (1). By the action of serine hydroxymethyltransferase (fig 1, enzyme 2), the β-carbon of serine is mobilized with the formation of 5,10-methylenetetrahydrofolate. Activation of formate by the formyltetrahydrofolate synthetase (enzyme 8) contributes 10-formyltetrahydrofolate to the pool of coenzymes. Interconversion of these two coenzyme forms occurs enzymically via 5,10-methenyltetrahydrofolate by cyclization of the 10-formyl-derivative (enzyme 7) and by dehydrogenation of the 5,10-methylene-derivative (enzyme 6). By this interrelated enzymic system (fig 1), the appropriate coenzymes for the synthesis de novo of purines and thymidylate are produced. It should be noted that tetrahydrofolate is a byproduct of purine biosynthesis, whereas dihydrofolate is formed concomitantly with thymidylate (enzyme 3). Thus, the persistent demand for dihydrofolate reductase (enzyme 4) is emphasized.

Especially important in the intestinal mucosa and storage tissues such as liver and kidney is the enzymic formation of 5-methyltetrahydrofolate (enzyme 5), necessary for the assimilation of dietary folates, for the transport and storage of folates. How 5-formyltetrahydrofolate, commonly called citrovorum factor[1] (CF) and used as calcium leucovorin in CF rescue (9), is converted following oral administration to the 5-methyl compound in the process of assimilation (10) is not completely understood. Although enzymic transformation of CF has been reported (11,12), the biochemistry involving the conversion of CF to the tetrahydrofolate coenzymes remains unclear. CF could enter the pool of coenzymes by several routes including deacylation or isomerization to 10-formyltetrahydrofolate.

METABOLIC REGULATION IN FOLATE METABOLISM

Throughout its history, folic acid (folate) has been studied in microbial systems with *Streptococcus faecium* var. *durans* (*Streptococcus lactis* R) and *Lactobacillus casei* (13,14). Of these folate auxotrophs, *S. faecium* var. *durans* (15) has been especially valuable in the investigation of the role of folate in the de novo synthesis of purines, thymine, and serine (16–18) and in the study of the mechanism of resistance to chemotherapeutic agents (18–20) as investigated with drug-resistant mutant strains (17).

Studies with the MTX-resistant mutant strain of *S. faecium* var. *durans*, designated SF/A_k, have shown that end products of folate metabolism control their own synthesis by repressing the synthesis of enzymes required for the formation of the appropriate coenzymes. Repression of the formyltetrahy-

[1]Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

drofolate synthetase by purines regulates synthesis of 10-formyltetrahydrofolate (21). Serine, having a unique regulatory position because it is both a major source of one-carbon fragments and also an end product of folate metabolism, apparently controls the synthesis of several enzymes differentially (22). Whereas the 5,10-methylenetetrahydrofolate dehydrogenase and the 5,10-methenyltetrahydrofolate cyclohydrolase are repressed more severely than serine hydroxymethyltransferase, formyltetrahydrofolate synthetase is positively induced. The strong repression of this dehydrogenase which catalyzes the interconversion of coenzymes at the formaldehyde and formate levels, the partial repression of serine hydroxymethyltransferase required for the utilization and biosynthesis of serine, and the concomitant positive induction of the formyltetrahydrofolate synthetase reflect the fine physiologic balance within this vital area of metabolism.

By contrast, exogenous folate supplied in the culture medium (23) acts as a positive inducer of the dihydrofolate reductase activity of this mutant (24). Data presented in table 1 indicate that microbial growth and enzyme synthesis are positively regulated by folate and that the synthesis of reductase does not proceed during exponential growth at a constant differential rate or specific activity. With folate, there is essentially a doubling of enzyme synthesis during the exponential phase of growth. The acceleration of enzyme synthesis during growth possibly reflects a de-repression concomitant with the exhaustion of an unknown repressor. Because thymine added exogenously with purines appears to repress synthesis of dihydrofolate reductase (and purines are not repressors [21]), a derivative of thymine might regulate synthesis of reductase. Although the nature of the negative effector is unknown, it appears to antagonize the positive induction by folate (table 1, column 5).

It should be noted that the total dihydrofolate reductase activity of SF/A_k consists of two types of dihydrofolate reductase. The *folate reductase* catalyzes the reduction of folate and dihydrofolate (mammalian type). The *specific dihydrofolate reductase* (bacterial type) catalyzes synthesis of tetrahydrofolate from dihydrofolate; folate is not a substrate. The folate reductase is eight times more abundant in SF/A_k. Whereas both enzymes are induced by folate, the folate reductase differs in being extremely labile after exponential growth ceases.

In an attempt to prevent the stationary phase decay of dihydrofolate reductase activity of SF/A_k (89% folate reductase) with a subinhibitory amount of MTX, it was observed that MTX apparently influenced the synthesis of the dihydrofolate reductase (table 2). In addition to partially preventing the stationary phase decay of enzyme activity by forming a drug-enzyme complex which dissociated upon dialysis at pH 8.5, MTX also increased the enzyme activity of exponential phase cells. Because data such as these described for cultures grown in the presence of a subinhibitory level of MTX from the onset of growth (table 2) were not obtained with cultures to which MTX had been added during the course of growth or at the end of exponential phase, it is possible that the apparent positive induction by MTX reflected a de-repression resulting from a decrease in the intracellular level of a repressor substance. Although not growth-inhibitory, MTX, at the level used, could have restricted the biosynthesis of a "folate-dependent" repressor. It is of interest that methasquin (25), the quinazoline anti-

TABLE 1.—Dihydrofolate reductase activity of SF/A_k during growth with different supplements*

Optical density of cultures†	Medium supplements‡			
	Folate (ng/ml)		Purines and thymine	Purines, thymine, and folate
	1	10		
0.1	0.24	0.34	0.12	0.25
0.2	0.38		0.29	
0.3	0.48§	0.47	0.32	0.39
0.4	0.50		0.43§	
0.5–0.6		0.62	0.36	0.42
0.9–1.0		0.58§		0.48§

*From ref 24.

†Individual 30-ml static cultures in 50-ml flasks were incubated at 37°C until indicated culture density was reached; cells were then harvested and washed and cell extracts were prepared for dihydrofolate reductase assay (30°C, with KCl). Enzyme activity is expressed as units/mg of protein.

‡Purines (adenine, guanine, and xanthine, each at 10 μg/ml); thymine (3 μg/ml); and folate (1 ng/ml, when added together with purines and thymine).

§Activity of cultures at end of exponential phase.

TABLE 2.—Effect of MTX on synthesis of dihydrofolate reductase activity of SF/A_k*

Description of culture†	Specific activity of undialyzed extracts	Specific activity of dialyzed extracts			
		pH 7.4		pH 8.5	
		1 day	4 days	1 day	4 days
Exponential phase					
Control	0.60	0.55	0.51	0.54	0.51
MTX-grown	0.85	0.81	0.73	0.77	0.77
Stationary phase					
Control	0.14	0.14	0.12	0.10	0.16
MTX-grown	0.32	0.32	0.32	0.49	0.49

*From ref 24.

†Cultures (600 ml) in a purine- and pyrimidine-free medium supplemented with 10 ng/ml of folate were harvested toward the end of exponential phase and 2 hrs after growth at exponential rate had ceased. Control: culture without MTX; MTX-grown: culture grown in medium with 1 μg/ml of MTX. Resultant extracts were assayed for dihydrofolate reductase activity (30°C, with KCl) on the day of preparation and after dialysis at 4°C in 10 mM potassium phosphate -1 mM EDTA, pH 7.4 buffer and in 50 mM potassium phosphate-1 mM EDTA, pH 8.5 buffer for the time indicated.

folate which binds reductase of SF/A_k more strongly than MTX, partially protected reductase from decay but failed to induce under conditions investigated.

Similar and more complex mechanisms of the regulation of folate metabolism have been described for cell systems other than this microbial auxotroph (26).

Current interest in the possibility of differences in the regulation of folate metabolism in normal mammalian cells and malignant systems has prompted studies in our laboratory with the murine leukemia ERLD in vitro (27) and the murine L1210 system in vivo. As observed with other cells in culture (28), CF is more effective than folate in promoting the maximum cell yield of ERLD in vitro. A tenfold quantitative difference exists in the optimal concentrations. Experimental probing has resulted in preliminary data which suggest that CF, also more effectively than folate, induced synthesis of the dihydrofolate reductase of ERLD. Similarly, CF apparently also stimulated higher levels of enzymes required to mobilize serine and convert 5,10-methylenetetrahydrofolate to 5,10-methenyltetrahydrofolate. The effects of CF administered orally to leukemic mice apparently differ. Leukemic mice typically suffer a wasting of the small intestine with a decrease in total weight and protein and partial loss of intestinal dihydrofolate reductase activity. Results of initial investigation suggest that folate (20 μg/ml) and CF (50 μg/ml) supplied in the water effected an increase in the level of intestinal reductase activity in leukemic mice without increasing the level of the leukemic reductase activity which is at least five times higher than the intestinal level. By contrast to results with ERLD, CF (as supplied) decreased the intestinal level of C-1 activating and interconverting enzymes in leukemic mice. It is noted that the levels of these enzymes in the liver, intestine, and spleen of leukemic mice were generally lower than the levels measured in normal tissues whereas the dihydrofolate reductase level of the leukemic spleen, reflecting the infiltration by leukemia cells, was twice the normal level.

Although these are initial observations, they are stimulatory information. It is possible that these results are suggesting that the synthesis of the folate enzymes of normal tissues is regulated by the specific tissue level of reduced folates. Stressed by the folate requirements of the "parasitic" leukemic population, the host tissues are gradually depleted of intracellular pools which eventually fall to levels far below effective inducer levels. Folate and CF at the amounts administered might have influenced only the intestinal level of folates resulting in the induction of intestinal reductase. The synthesis of the reductase of L1210, apparently maximally induced to nearly twice the liver enzyme level by the folates derived from the host, was not further inducible by the folates administered. By contrast, several enzymes of the ERLD leukemia in vitro were apparently induced by CF. Perhaps CF, more effectively than folate, yields the appropriate inducer substance which is available to L1210 from the outset of leukemic proliferation in mice. Thus, it is also possible that these initial comparative studies emphasize the intricacies of enzyme regulation within a nutritionally competitive malignant cell system in vivo which are completely abolished by the cultivation of a single cell type in vitro (29).

METABOLIC REGULATION DURING ANTIFOLATE THERAPY-CF RESCUE

Obviously, the initial physiologic effect of antifolates, prevention of the synthesis of tetrahydrofolate coenzymes, leads to the cytotoxic effect, block-

ade of nucleic acid biosynthesis. Indirectly, antifolates possibly also affect the natural regulatory mechanisms which control the synthesis and activity of enzymes required for the synthesis of tetrahydrofolate coenzymes. By restricting biosynthesis of purines, thymidylic acid, serine, and methionine (26), antifolates do indeed lower intracellular pools of these metabolites. Under normal conditions of physiologic homeostasis, these end products, when present intracellularly in excess of the metabolic requirement, can be viewed as beneficial regulators, functioning as antimetabolites, according to an economical and purposeful design, to prevent the over-synthesis of enzymes and unrequired metabolites. Under conditions of antifolate therapy, proliferating neoplastic cells, having a great demand for precursor metabolites, exhaust their metabolic pools, causing death. For normal cells in which demands for cell division are markedly lower, the antifolate block could have a differential effect dependent upon the maintenance of depressed levels of metabolite pools. In such antifolate-stressed normal cells with a severely restricted metabolism, the response to the decreased metabolite level could be one of enzyme de-repression. Thus, when CF is administered orally after MTX to rescue the normal cells and is subsequently converted to 5-methyltetrahydrofolate during assimilation, a full complement of activating and interconverting enzymes would be available to synthesize other tetrahydrofolate coenzymes and implement them efficiently in biosynthetic pathways.

CF rescue by itself or primed by enzyme derepression would require that cells synthesize methionine from 5-methyltetrahydrofolate and homocysteine. On the assumption that there is essentially no conversion of the 5-methyl-derivative to 5,10-methylenetetrahydrofolate, methionine synthetase would be necessary for CF rescue.

In pondering possible explanations for CF rescue, perhaps it is not bizarre to speculate that CF, after its conversion to 5-methyltetrahydrofolate, tetrahydrofolate, and dihydrofolate, also represses and/or positively induces enzymes differentially in normal and neoplastic cells.

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Comparative Effects of Folate Antagonists Versus Enzymatic Folate Depletion on Folate and Thymidine Enzymes in Cultured Mammalian Cells [1]

P. L. Chello,[2,3,*] C. A. McQueen,[2] L. M. DeAngelis,[2] and J. R. Bertino [2]

SUMMARY

Folate and thymidine enzymes were monitored during lag, log, and stationary growth of RPMI 4265 human lymphoblasts. The activities of dihydrofolate reductase, thymidylate synthetase, thymidine kinase, thymidylate kinase, and methionine synthetase peaked during log phase, while those of serine transhydroxymethylase and 5,10-methylenetetrahydrofolate reductase were maximum during stationary phase. 10-Formyltetrahydrofolate synthetase had peaks of activity in log and in stationary phase, while the activity of 5,10-methylenetetrahydrofolate dehydrogenase did not change during the growth cycle. In cells whose growth was inhibited with folate antagonists (methotrexate, chlorasquin, or triazinate), dihydrofolate reductase was inhibited and thymidylate synthetase and thymidine kinase were elevated twofold and threefold respectively. Other enzymes were unaffected. Growth with noninhibitory concentration of each antifolate affected only dihydrofolate reductase activity (inhibition when assayed at pH 7.0; elevated activity in extracts from methotrexate- and chlorasquin-treated cells when assayed at pH 8.5). The folate-cleaving enzyme, carboxypeptidase G_1, produced intracellular folate depletion. In the presence of 0.0025 units/ml, growth inhibition occurred after the cells reached mid-log phase. The activities of dihydrofolate reductase, thymidylate synthetase, and thymidine kinase were significantly lower 24 hours before, and were undetectable when growth inhibition occurred. In contrast, the activities of other enzymes were not reduced until growth inhibition occurred. Use of 0.001 units/ml also produced an intracellular folate depletion, but did not significantly retard growth. In these cells, only the activity of dihydrofolate reductase was lower than in control cells.

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The importance of folate[4] coenzymes for purine, pyrimidine, and amino acid biosynthesis is well documented, and the enzymes responsible for the formation and utilization of these coenzymes have been established (1). However, little is known about the interaction and regulation of these enzymes in the cell, and that which is known has been determined from a variety of sources, predominantly bacterial (2). For a better understanding of the folate biochemistry of mammalian cells, we utilized the RPMI 4265 human lymphoblast in culture. These cells had a pattern of dihydrofolate reductase activity directly related to the stage of growth (3,4) which facilitated studies of the patterns of other folate enzymes and related thymidine enzymes.

Herein we present a comparative study of the effect of folate antagonists versus folate depletion on enzymatic patterns within these cells. Three classes of 2,4-diaminofolate antagonists were investigated: (*a*) the established, clinically employed pteridine derivative, methotrexate; (*b*) a potent quinazo-

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[4] The term folate is used to denote pteroylglutamate and its reduced, substituted, and conjugated derivatives.

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line derivative, chlorasquin (5–7); and (*c*) a triazine derivative, triazinate (Baker's antifol), which is being investigated in both the laboratory (8–10) and the clinic (11,12).[5] Because of the potential of enzyme therapy, folate depletion was produced by a folate-degrading enzyme, carboxypeptidase G_1 (13), which has chemotherapeutic efficacy against various tumor lines carried in culture (14,15) and in animals (15), and is undergoing clinical trials (16).[6] Parts of this study have been published (4,17–20).

MATERIALS AND METHODS

RPMI 4265, a line of immature lymphoblastoid leukocytes established in culture after isolation from a patient with chronic myelocytic leukemia (21), was obtained from the Roswell Park Memorial Institute, Buffalo, NY. The cells were grown in suspension culture in RPMI 1640 medium (Grand Island Biological Co, Grand Island, NY) containing 15% fetal calf serum (Flow Laboratories, Rockville, Md).

Methotrexate was purchased from Lederle Laboratories, Pearl River, NY. Chlorasquin and triazinate were supplied by Dr. Florence White, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. All solutions were prepared in resin-distilled water and sterile-filtered with 0.22-μm Swinnex (Millipore) filters. Carboxypeptidase G_1, purified from *Pseudomonas stutzeri* (13), was diluted with 0.5 M Tris-HCl, pH 7.5, and filtered with 0.22-μm Swinnex filters. One unit of activity was defined as that amount of enzyme which hydrolyzed 1 μmol of substrate per minute at 37°C (13).

Cells in late logarithmic growth were harvested and resuspended at 1.25–2.0 $\times$ 10^5 cells/ml. After addition of drug or enzyme, cells were dispensed into 150-ml sterile GIBCO serum bottles, 50 ml/bottle, and incubated at 37°C. At 12- and 24-hour intervals, cells were counted and harvested by centrifugation at 4°C. Cell counts from bottles within an experimental group differed no more than 10%. Sufficient cells were harvested from each experimental group to yield duplicate cell packs containing 30–45 $\times$ 10^6 cells. These cell packs were stored at −68°C for later determination of enzymatic activity. The enzymes to be studied were stable for periods of at least 3 months.

Crude enzyme extracts were prepared by thawing the cell packs in 0.05 M Tris-HCl buffer, pH 7.5, at a density of 25–30 $\times$ 10^6 cells/ml. The cells were disrupted by sonic oscillation and the sonicate was centrifuged at 37,000 *g* for 20 minutes in a Sorval RC-2 to remove cell debris. All procedures were carried out at 0–4°C. The supernatant was assayed for enzyme activity by established procedures (4,22–29). Since the activities of several enzymes were monitored in each extract, the activity of dihydrofolate reductase and/or thymidylate synthetase was always determined as a control. Protein content of the crude preparations was determined by a microbiuret assay (30).

Intracellular folate levels were determined by *Lactobacillus casei* assay (31) using cell suspensions grown in exactly the same manner as for enzyme monitoring.

RESULTS

The growth profile of RPMI 4265 cells is shown in figure 1. Following a 24-hour lag, the cells divided exponentially for 72 hours after which they entered a stationary or resting phase which was monitored for 48 hours. Cells could remain in stationary phase for an additional 48 hours without apparent loss of viability. With one exception, the activity of each enzyme changed during growth (fig 1). Dihydrofolate reductase and thymidylate synthetase had identical patterns of activity but a tenfold difference in specific activity. Each increased to a fourfold to fivefold peak at mid-log phase and decreased as the cells entered the later stages of growth. The peak of either enzyme served as a marker for mid-log phase. Methionine synthetase (5-methyltetrahydrofolate-homocysteine transmethylase) and thymidine kinase were also log-phase enzymes, with peaks of activity in early and late log respectively. The specific activity of methionine synthetase was the lowest of all the enzymes examined, while that of thymidine kinase was comparable to thymidylate synthetase. Thymidylate kinase doubled in activity by late log. Throughout the growth cycle the specific activity of thymidylate kinase was greater than the combined specific activities of thymidine kinase and thymidylate synthetase.

Serine transhydroxymethylase and 5,10-methylenetetrahydrofolate reductase also increased during the log phase. However, in contrast to dihydrofolate reductase and other enzymes, the activity of each remained at a maximum into early stationary phase. The specific activity of serine transhydroxymethylase was in the same range as dihydrofolate reductase, while the specific activity

[5] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.
Chlorasquin: NSC-529861; aspartic acid, *N*-[*p*-[[(2,4-diamino-5-chloro-6-quinazolinyl)methyl]amino]benzoyl]-, dihydrate, L-.
Triazinate: NSC-139105; CAS reg. No. 41191-04-2; ethanesulfonic acid, compd. with α-[2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazin-1(2*H*-yl)phenoxy]-*N*,*N*-dimethyl-*m*-toluamide (1:1).

[6] Carboxypeptidase G_1: CAS reg. No. 9054-73-3.

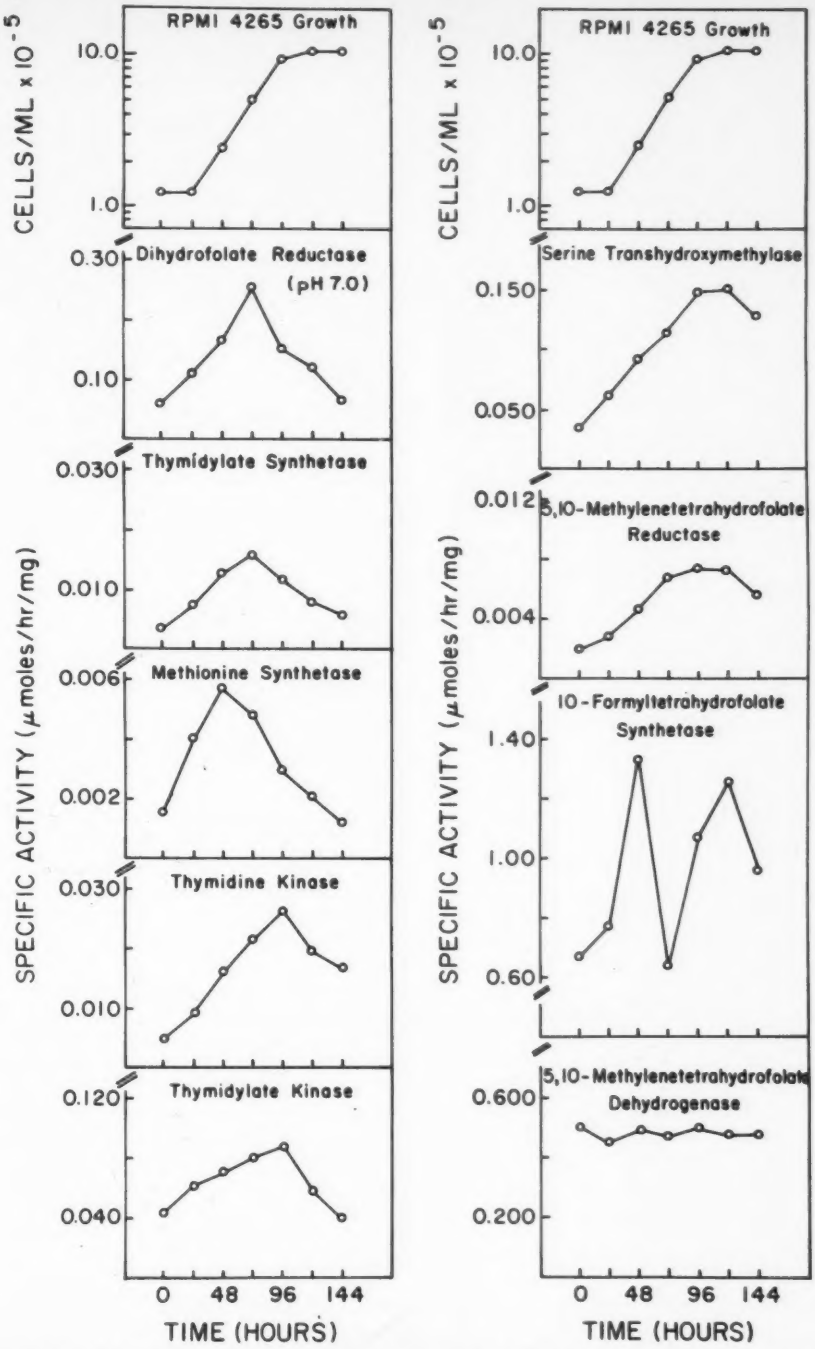

FIGURE 1.—Enzyme activity in RPMI 4265 cells. RPMI 4265 cells were diluted into fresh medium and grown for 144 hrs. At 0 time and at 24-hr intervals, cells were counted and duplicate cell packs were harvested. Each point represents the mean enzymatic activity from the duplicate cell packs. Each enzyme profile has been verified at least 3 times.

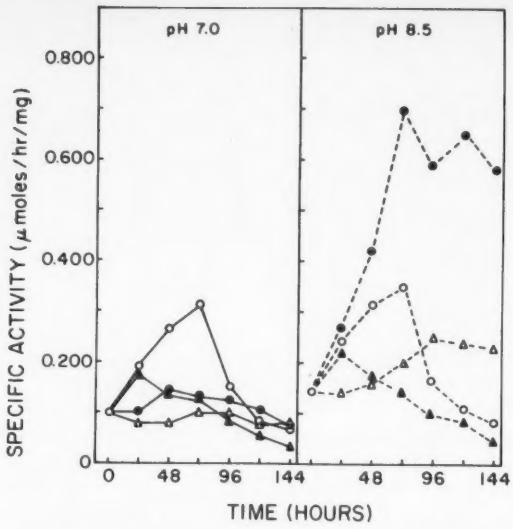

FIGURE 2.—Dihydrofolate reductase activity in RPMI 4265 cells grown with 10^{-8} M methotrexate, chlorasquin, or triazinate. RPMI 4265 cells were diluted into fresh medium containing either no drug (○), 10^{-8} M methotrexate (●), 10^{-8} M chlorasquin (△), or 10^{-8} M triazinate (▲). Duplicate cell packs were harvested at 0 time and at 24-hr intervals for 144 hrs. Dihydrofolate reductase was extracted and assayed at pH 7.0 (—) and at pH 8.5 (----). Each point represents the mean enzymatic activity determined in the duplicate cell packs. This experiment has been repeated 3 times with similar results.

of 5,10-methylenetetrahydrofolate reductase and methionine synthetase was low. The activity patterns of 10-formyltetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase were unique. The former had two peaks of activity: the first during early log growth and the second in early stationary phase. The specific activity of the latter showed no significant change during the growth cycle. The specific activity of each was higher than the specific activity of dihydrofolate reductase.

The concentration of each antifolate which produced a 50% inhibition of growth at 96 hours was 5.9×10^{-8} M, 4.7×10^{-8} M, and 9.0×10^{-8} M for methotrexate, chlorasquin, and triazinate respectively. At 10^{-8} M, neither antagonist caused more than a 5% retardation of growth, but each had a pronounced effect on the activity of dihydrofolate reductase (fig 2). The reductase activity in control cells, determined at pH 7.0 or pH 8.5, increased to the expected peak at mid-log phase and then declined. A slight activation was evident when the pH of the assay was increased to 8.5. The pattern of dihydrofolate reductase activity in drug-treated cells, when assayed at pH 7.0, did not change significantly during the growth cycle. The activity was about one third of that of control cells at mid-log phase. When the reductase activity was assayed at pH 8.5, a different pattern occurred with each antifolate. In cells grown with 10^{-8} M methotrexate, enzyme activity rose to a level twice that of the controls by mid-log and remained elevated throughout the remainder of the growth cycle. In cells exposed to 10^{-8} M chlorasquin, the assay at pH 8.5 revealed an increase in activity only in cells harvested at the later stages of growth. This increase was not of the same magnitude as that found in cells grown with methotrexate. At pH 8.5, no change in enzymatic activity was detected in the extracts from cells grown with triazinate. At 10^{-8} M, the antifolates had no effect on the activities of the other enzymes.

Higher levels of antifolate produced the growth profiles and the changes in enzymatic activity shown in figure 3. At 10^{-7} M, methotrexate or chlorasquin completely inhibited growth. Triazinate was

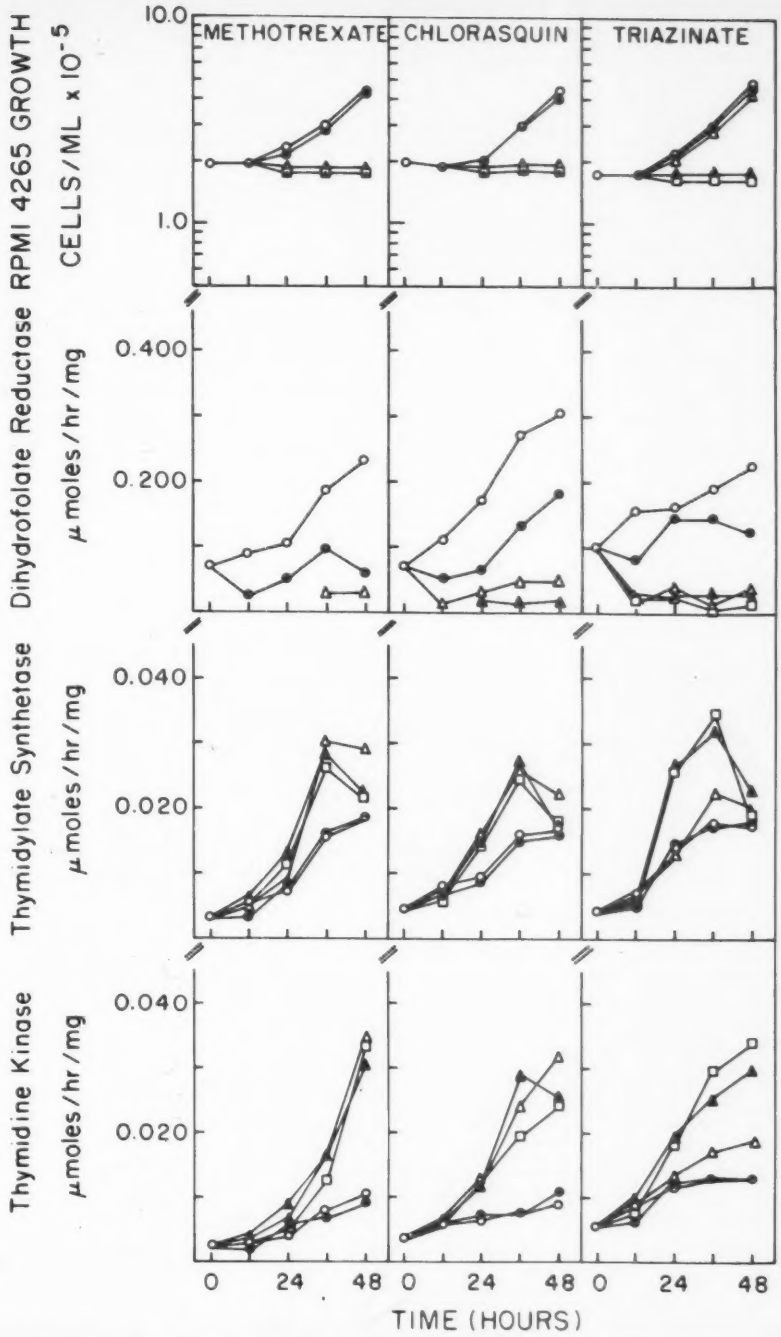

FIGURE 3.—Growth and enzyme activity of control and antifolate-treated cells. RPMI 4265 cells were grown for 48 hrs with no drug (○) or 10^{-8} M (●), 10^{-7} M (△), 10^{-6} M (▲), and 10^{-5} M (□) antifolate. At 12-hr intervals, the cell number was monitored and duplicate cell packs were harvested. The vertical columns represent separate experiments. All enzymes were measured within the same experiment. Each point represents the mean enzymatic activity in the duplicate cell packs. Each experiment was repeated at least once with similar results.

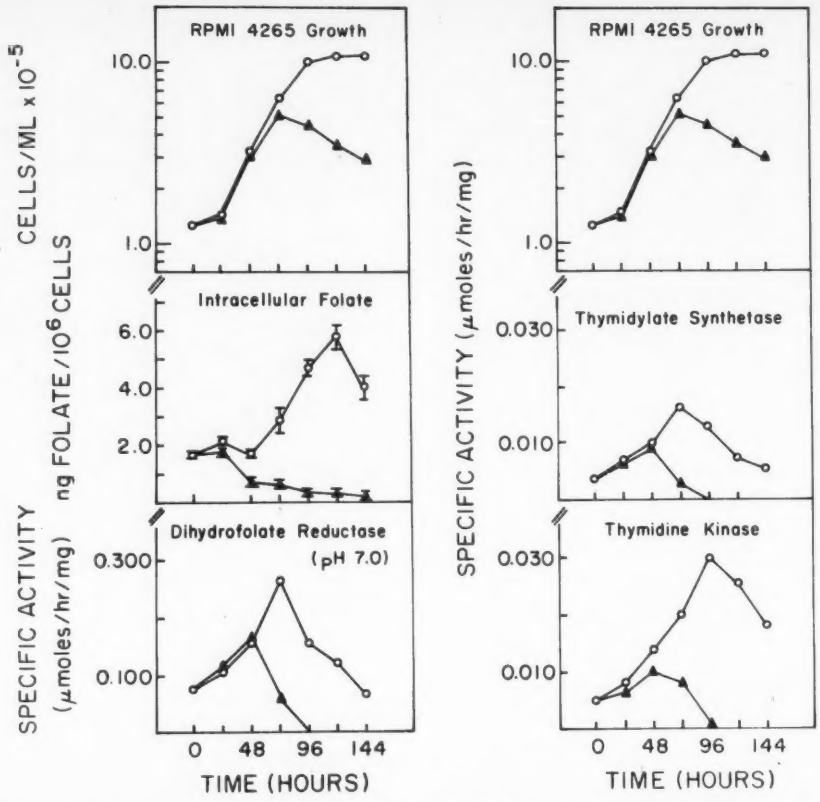

FIGURE 4.—Folate levels and enzyme activity in RPMI 4265 cells grown with 0.0025 units of carboxypeptidase G_1/ml of medium. RPMI 4265 cells were set up in the medium with (▲—▲) and without (○—○) 0.0025 units/ml of carboxypeptidase G_1. At 0 time and at 24-hr intervals, duplicate cell packs were harvested for the determination of folate levels and enzyme activity. A representative growth profile is shown at the top. The intracellular folate levels are the average of 3 experiments. Vertical bars indicate the standard deviation of the geometric mean. Each enzyme profile is a representative obtained at least 3 times.

less powerful; a level of 10^{-6} M was necessary for complete inhibition of growth. Dihydrofolate reductase activity was progressively inhibited as the concentration of antifolate was increased. Reassay at pH 8.5 (not shown) again revealed elevated levels in the methotrexate- or chlorasquin-treated cells, but not in the triazinate-treated cells. Thymidylate synthetase activity in control cells increased approximately threefold during growth. When growth was completely inhibited by any of the folate antagonists, thymidylate synthetase activity underwent an additional twofold elevation by 36 hours and declined thereafter. In control cells or in cells grown with 10^{-8} M antifolate, thymidine kinase activity increased threefold to fourfold in 48 hours. Antifolate concentrations which completely inhibited growth elevated the thymidine kinase activity an additional threefold by 48 hours. The three antifolates did not elevate the activities of the other enzymes under investigation.

The effect of 0.0025 units of carboxypeptidase G_1/ml of medium on RPMI 4265 growth and intracellular folate levels is shown in figures 4 and 5. Growth of cells with carboxypeptidase G_1 approximated that of control cells through mid-log (72 hours), after which a drastic and complete inhibition accompanied by cell loss occurred at later times. The level of intracellular folates in control cells, expressed as ng folate/10^6 cells, increased in log phase to a maximum during stationary phase. In enzyme-treated cells, a progressive intracellular depletion was evident during both log and stationary phases. Enzyme activity in these cells conformed to two patterns: (*a*) those whose activity was affected prior to the inhibition of growth; and (*b*) those whose activ-

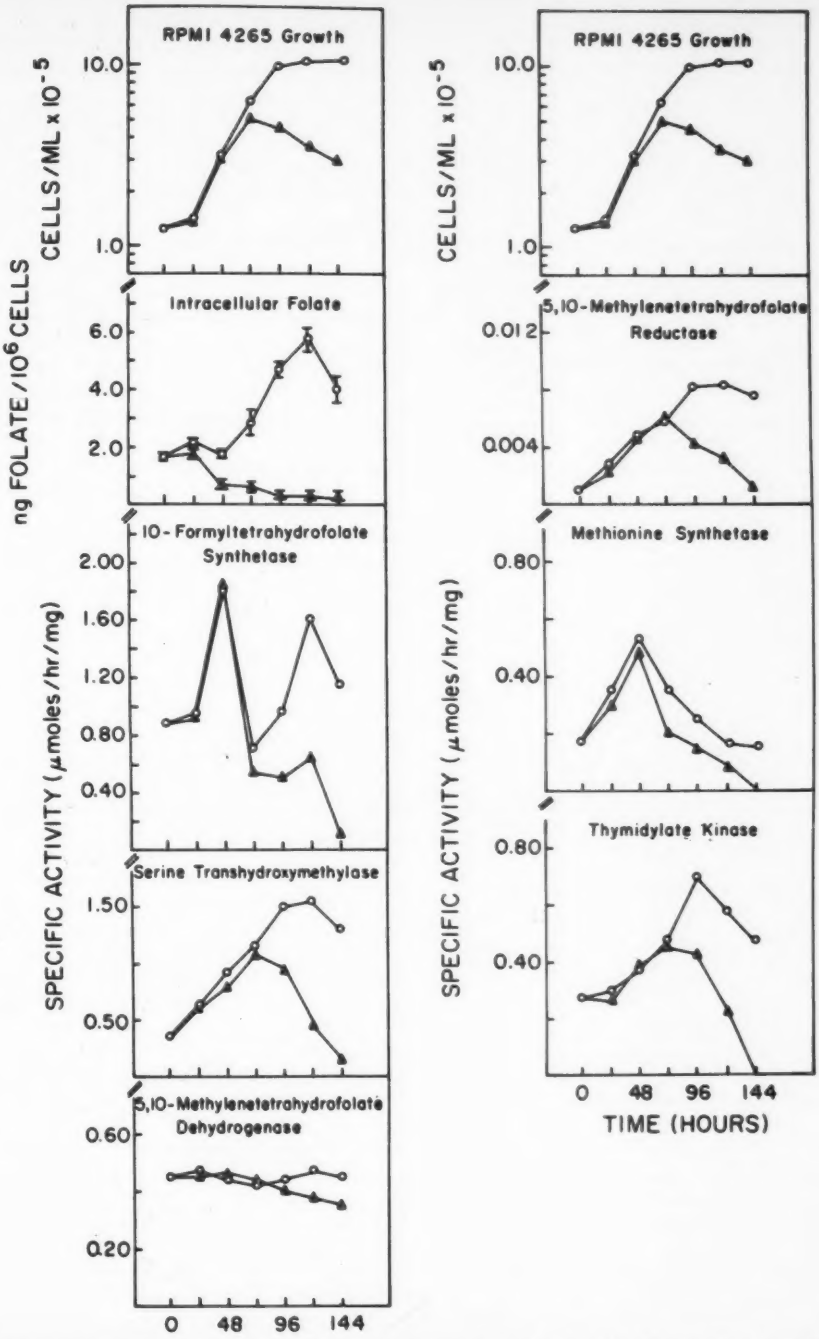

FIGURE 5.—Folate levels and enzyme activity in RPMI 4265 cells grown with 0.0025 units of carboxypeptidase G_1/ml of medium. RPMI 4265 cells were set up in medium with (▲—▲) and without (○—○) 0.0025 units/ml of carboxypeptidase G_1. At 0 time and at 24-hr intervals, duplicate cell packs were harvested for the determination of folate levels and enzyme activity. A representative growth profile is shown at the top. The intracellular folate levels are the average of 3 experiments. Vertical bars indicate the standard deviation of the geometric mean. Each enzyme profile is a representative obtained at least 3 times.

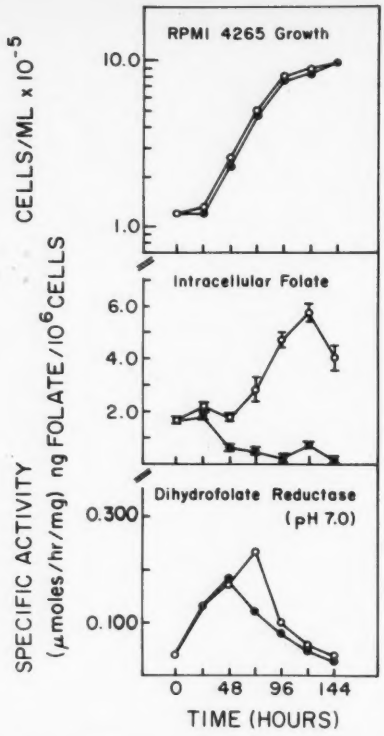

FIGURE 6.—Folate levels and dihydrofolate reductase activity in RPMI 4265 cells grown with 0.001 units of carboxypeptidase G_1/ml of medium. RPMI 4265 cells were set up in medium with (●—●) and without (○—○) 0.001 units/ml of carboxypeptidase G_1. At 0 time and at 24-hr intervals, duplicate cell packs were harvested for the determination of folate levels and enzyme activity. A representative growth profile is shown at the top. The intracellular folate levels are the average of 3 experiments. Vertical lines indicate the standard deviation of the geometric mean. The dihydrofolate reductase profile is a representative obtained > 6 times.

ity was affected with the inhibition of growth. The first group, shown in figure 4, consisted of dihydrofolate reductase, thymidylate synthetase, and thymidine kinase. The activity of each was significantly lower ($P < 0.05$) than control activity at mid-log phase (72 hours) and was undetectable when growth ceased. In the second group, shown in figure 5, activity decreased coincidentally with the inhibition, but was detectable throughout the remainder of the experiment.

When cells were exposed to less carboxypeptidase G_1 (0.001 units/ml), intracellular folates were depleted without a corresponding inhibition of growth (fig 6). With the exception of dihydrofolate reductase, enzyme patterns were identical to those in control cells. Dihydrofolate reductase activity increased in the carboxypeptidase G_1 cells during the lag and early-log phase (48 hours), but the expected peak at mid-log (72 hours) was significantly lower ($P < 0.05$) than in the controls. Reductase activity at later time points was not significantly different from that in control cells.

DISCUSSION

Since 0.001 units/ml of carboxypeptidase G_1 decreased dihydrofolate reductase activity, while 10^{-8} M antifolate elevated the total cellular reductase content, these data suggest that folate stabilizes the enzyme. Previous clinical and laboratory studies (3,32–34) have suggested that methotrexate protects the reductase enzyme from degradation in vivo either by virtue of its binding, or indirectly through accumulation of a folate coenzyme such as dihydrofolate (monoglutamate or polyglutamate). Our studies do not indicate which mechanism prevails, but do show that the effect can be produced by another class of antifolates, as represented by chlorasquin. The chlorasquin elevation was not comparable to that produced by methotrexate, presumably because the tighter binding of the quinazoline at pH 8.5 (5,7) allowed less unmasking of inhibited enzyme. Enzyme elevation was not found in extracts from cells grown with triazinate, but can be explained by the uniform binding of this inhibitor over a broad pH range (4) and our inability to dissociate the enzyme-triazinate complex. An alternative explanation for the enzyme elevation during growth with folate antagonists is that the amount of free enzyme regulates its own synthesis, ie, with less free enzyme, more enzyme is synthesized and subsequently inhibited. However, the loss of reductase activity during carboxypeptidase G_1 folate depletion supports the stabilization hypothesis. As further support, the work of Jackson and Huennekens (35) showed a slower turnover of dihydrofolate reductase in methotrexate-treated mammalian and bacterial cells. Finally, although in vivo mechanisms of stabilization and degradation may be different, there is ample evidence that folate and folate antagonists protect dihydrofolate reductase from denaturation in vitro (36–38). To summarize, these data are consistent with a control of the level of dihydrofolate reductase through cofactor stabilization. The level of enzyme is increased when an analog is present and decreased when folate pools are reduced.

Concentrations of either folate antagonist which inhibit growth cause an elevation of thymidylate synthetase and thymidine kinase, as well as an increase in the total amount of dihydrofolate reductase. The methotrexate elevation of these enzymes

has been reported in regenerating rat liver (39,40) and in human (41–44) and animal (45,46) cells in culture. Since methotrexate can inhibit thymidylate synthetase in vitro (4,44,47), the in vivo elevation has been attributed to enzyme stabilization (44,46). However, recent studies have shown that triazinate, which also elevates thymidylate synthetase in vivo, does not inhibit the enzyme in vitro (4). Therefore, triazinate would presumably not bind to the synthetase enzyme in vivo to stabilize it. In addition, other data show that methotrexate, chlorasquin, and triazinate do not adequately protect thymidylate synthetase from heat denaturation in vitro (4,46). With regard to thymidine kinase, the elevation in RPMI 4265 cells cannot be related to in vitro inhibition or stabilization by the folate antagonists (4).

Since the antifolates (especially triazinate) cannot be held directly responsible for the elevation of these thymidine enzymes, alternative explanations are that a folate coenzyme acts as an inducer or as a stabilizer. Folic acid has been documented to induce dihydrofolate reductase synthesis in bacteria (48–51), and Hillcoat et al (52) have reported that an increase in dihydrofolate reductase activity dependent upon RNA and protein synthesis occurs when RPMI 4265 cells are grown with high concentrations (10^{-4} M) of folic acid. If folate is considered in an inductive role, the loss of thymidylate synthetase activity and thymidine kinase activity in conjunction with the loss of dihydrofolate reductase activity at 0.0025 units/ml of carboxypeptidase G_1 is understandable. However, it is also possible that folate pools such as dihydrofolate (as monoglutamate or polyglutamate) are rearranged and function as stabilizers. Although in vitro heat stability is only an approximation of in vivo degradative processes, such studies have shown that dihydrofolate is a poor protector of thymidylate synthetase (4,46), but the combination of deoxyuridine monophosphate and dihydrofolate is effective (46). A third possibility is that changes in the folate pools modify thymidine pools, with corresponding changes in the levels of both thymidylate synthetase and thymidine kinase, as suggested by others (42,44,45).

To summarize, a decrease in folate pools as a consequence of enzymatic folate depletion results in reduced dihydrofolate reductase activity, thymidylate synthetase activity, and thymidine kinase activity, while an alteration in these pools by folate antagonists leads to increased total amounts of all three enzymes.

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Effects of Methotrexate on Cellular Metabolism: Some Critical Elements in the Drug-Cell Interaction [1,2]

I. David Goldman [3,*]

Recent studies from several laboratories employing diverse experimental approaches have demonstrated the requirement for the continuous exposure of mammalian cells to methotrexate (MTX) to achieve the metabolic effects of this agent in vitro and cytotoxicity in vivo (1–9). The basis for these observations has been related to the critical requirement for free intracellular MTX in the suppression of tetrahydrofolate (THF) synthesis and THF-dependent processes within the intact cell (3–7,10). The inherent limitation in the capacity of mammalian cells to accumulate free MTX has been documented (11,12). Finally, the role of free intracellular drug as a critical pharmacologic endpoint in the treatment of human malignancies has been suggested by the increased potency of high-dose MTX regimens which sustain high extracellular MTX concentrations over long intervals, conditions which should optimize accumulation of free intracellular drug (7,13–16).

Based upon new information regarding the pharmacologic endpoints for MTX (7) and the success of treatment regimens directed against extracerebral tumors, this Workshop has dealt with the possible role for high-dose MTX regimens in the treatment of central nervous system malignancies. The characteristics of the permeability properties of the blood-brain barrier and the choroid plexus provide a unique opportunity to generate high levels of MTX within the cerebrospinal fluid compartment after intrathecal infusion of drug while minimizing exposure of susceptible tissues bathed by systemic fluids to this agent (17). In this article, recent concepts on the mechanism of action and pharmacokinetics of MTX on a cellular level are reviewed. The biochemical basis for the role of free intracellular MTX as an important cytotoxic determinant is developed and apparent discrepancies between the effects of MTX in vivo and in vitro are reconciled.

Critical Role for Free Intracellular MTX in the Suppression of (*a*) THF Synthesis and (*b*) THF-Dependent Pyrimidine, Purine, and Amino Acid Synthesis

When L-cell mouse fibroblasts are loaded to intracellular MTX levels in excess of the apparent capacity of high-affinity binding sites, following which free drug is eliminated from the intracellular and extracellular compartments, while a large portion of total drug appears to remain essentially irreversibly bound within the cell, incorporation of ^{3}H-deoxyuridine (^{3}H-UdR) or ^{14}C-formate into DNA is only minimally perturbed (3,5). To achieve complete inhibition of these processes requires sustained exposure of cells to extracellular MTX with the generation of intracellular drug in excess of the capacity of high-affinity binding sites (exchangeable MTX) with a concentration of exchangeable drug many orders of magnitude above the K_i for dihydrofolate reductase (DHFR) (3,5). While a number of investigators have attributed apparent competitive interactions between MTX and 5-formyl-THF (citrovorum factor) in vivo, and the requirement for free intracellular MTX in vitro, to the ability of MTX to inhibit thymidylate synthetase (9,18,19), studies from this laboratory have shown that this requirement for exchangeable intracellular MTX is not related solely to the inhibition of THF-dependent thymidylate synthesis. Hence, exchangeable intracellular drug is also required to suppress ^{14}C-formate incorporation into RNA and protein in the L-cell mouse fibroblast, suggesting that MTX inhibits a process common to the three biosynthetic pathways. Finally, the requirement for free intracellular MTX in the suppression of THF-dependent processes can now be accounted for both theoretically (10) and experimentally (6,7) by the critical role of this intracellular MTX component in the inhibition of the reduction of DHF to THF. Association of MTX with high-affinity binding sites alone in the absence of exchangeable drug does not result in inhibition of DHF reduction to THF in the Ehrlich ascites tumor.

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[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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Rather, inhibition of this process requires sustained exposure of cells to MTX, with 50% suppression of DHF reduction at an exchangeable intracellular MTX level of ~0.15 μM and complete suppression achieved only when exchangeable intracellular MTX is in excess of ~5 μM (fig 1; see also ref 6), a value five orders of magnitude above the K_i for DHFR in this cell line (1.6×10^{-11} M at pH 7.2). Hence, sufficient DHFR activity must persist within cells to sustain near normal rates of THF synthesis until appreciable levels of free MTX accumulate within the intracellular water.

Biochemical Basis for the Requirement for Free Intracellular MTX in the Suppression of THF Synthesis

Unlike folic acid, DHF is an excellent substrate for DHFR and is rapidly reduced to THF as it is generated during the synthesis of deoxythymidylate from deoxyuridylate. Indeed, the capacity for DHF reduction is so great that when Ehrlich ascites tumor cells are exposed to 10 μM ^{3}H-DHF, only THF can be identified within the cell; the intracellular DHF level is so low (< 10^{-8} M) as to be undetectable (6). Hence, since the K_m for DHF is ~1 μM, DHFR must be at a very low state of saturation with respect to its substrate and only a small percentage of total DHFR activity is required to meet cellular demands for THF. Accordingly, when cells are exposed to MTX and the drug enters the intracellular compartment, the following sequence of events would be expected to occur. Initially, as MTX binds to DHFR, free drug does not accumulate within the cell since influx is rate limiting to binding (11) and the ratio of free enzyme to intracellular drug is very great. Association of MTX with DHFR sites results in a small transient reduction in the rate of THF synthesis with a consequent increase in the DHF substrate level. Since the DHF level in the cell is initially so much lower than its K_m, the interaction between the DHF and DHFR is essentially first order and the increase in the intracellular concentration of DHF results in a proportional increase in the rate of DHF reduction as the net rate of interaction between the higher level of DHF and those enzyme sites unassociated with MTX increases. Hence, THF synthesis is initially maintained at an essentially normal rate as MTX associates with an increasing proportion of DHFR sites and DHF continues to accumulate within the cell. Ultimately, as MTX binds to the major portion of DHFR sites, the DHF concentration within the cell approaches its K_m, the rate of THF synthesis no longer increases in proportion to the increase in the intracellular DHF level, and the rate of THF synthesis falls. Also,

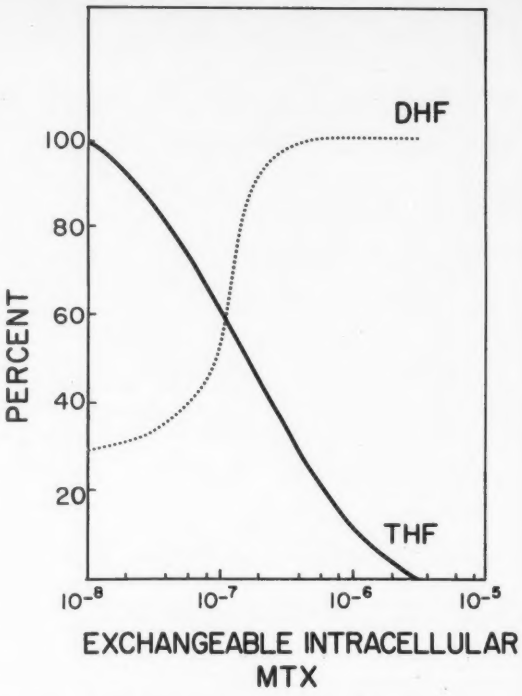

FIGURE 1.—Effects of exchangeable intracellular MTX on the metabolism of ^{3}H-DHF. Ehrlich ascites tumor cells were exposed to 10^{-5} M MTX for 30 mins, then separated from the medium and incubated for 1 hr in fresh media at extracellular MTX concentrations ranging from 10^{-8} to 10^{-5} M. The cells were then exposed to 10 μM ^{3}H-DHF for 15 mins following which sonic extracts were chromatographed. The data are plotted as a percentage of the THF level when exchangeable intracellular MTX is 10^{-8} M, a concentration which negligibly affects the cellular THF level, and as a percentage of the DHF level when exchangeable intracellular MTX is ~5 μM, a concentration at which the cellular DHF level is maximum. (*From* ref 6.)

because the interaction between MTX and DHFR is not "stoichiometric" at physiologic pH (20) and because cellular DHF has risen to levels so high that it competes with MTX for binding to the few remaining DHFR sites, free MTX accumulates within the cell at intracellular drug levels *below* molar equivalence to the DHFR content. Finally, in order to fully saturate DHFR sites and completely suppress THF synthesis, levels of free intracellular MTX orders of magnitude above its K_i are required. Conversely, when cells which contain sufficient free MTX to abolish THF synthesis are suspended into drug-free media, as exchangeable MTX leaves the cells under conditions in which the cellular DHF

/el is high, DHF displaces MTX from a few percent of the total DHFR sites before resumption of DHF reduction decreases cell DHF to trivial levels once again. Cellular MTX finally falls to a constant level which appears tightly bound within the cell but represents a value which is *less than* the total DHFR binding capacity. The concurrent rapid recovery of THF synthesis during efflux of exchangeable intracellular MTX leads to the interpretation that this is related to dissociation of MTX from a low-affinity form of DHFR while the phase of tight MTX binding within the cell represents interaction between the drug and a high-affinity form of the enzyme. However, this phenomenon may be accounted for entirely on the basis of alterations in the DHF level within the cell with competition between MTX and DHF for a small portion of a single high-affinity DHFR species.

In an extensive quantitative analysis of this phenomenon, Jackson and Harrap predicted that 95% of total DHFR sites within exponentially growing L1210 leukemia cells could be inhibited before the cellular growth rate is appreciably reduced (10). This prediction was made utilizing a K_i for MTX of 1.9×10^{-11} M, a value almost identical to that determined for the Ehrlich ascites tumor. As discussed further below, it would be expected that an even smaller percentage of DHFR activity would be sufficient to meet cellular demands for THF in slow-growing cell populations.

While the requirement for free intracellular MTX can be accounted for by the interaction between the drug and a single high-affinity DHFR species within the cell, this may be related, in addition, to the interaction between MTX and a low-affinity form(s) of DHFR. Multiple enzyme forms have been isolated from bacterial and mammalian cells (21–24) and in one case the affinity for MTX was shown to be reduced (23). The affinity of DHFR for MTX is considerably reduced in the absence of NADPH (25,27). Rothenberg suggested that forms of DHFR with a reduced affinity for MTX and decreased capacity to reduce DHF are present in L1210 leukemia cells at physiologic pH (28). It has not been possible as yet to demonstrate biochemically that forms of DHFR with a reduced affinity for MTX are present in the cells studied in this laboratory. This may not be technically feasible since isolation procedures may produce conditions considerably different from those of the intracellular compartment resulting in conversion of heterogeneous enzymes present within the cell to a homogeneous form in the cell-free system. Further, such enzyme forms might not be distinguishable from the major DHFR species since they might represent only a few percent of total enzyme within the cell. However, as pointed out above, even a low level of such an enzyme form(s) could play a major role in sustaining DHF reduction in the absence of appreciable levels of free MTX within the cell.

Factors That Alter the Requirement for Free Intracellular MTX in the Suppression of THF-Dependent Processes

According to the formulation described in the preceding section, the small percentage of DHFR activity required to meet cellular demands for THF along with the rising levels of DHF within the cell as DHFR sites are associated with MTX results in essentially normal rates of THF synthesis until the major portion of the enzyme is associated with MTX. It follows from this that the lower the "basal" rate of cellular deoxythymidylate synthesis from deoxyuridylate (*a*) the lower the cellular DHF level, (*b*) the smaller the fraction of total enzyme that is required to sustain THF cofactor levels in the absence of MTX, (*c*) the greater the degree of saturation of DHFR sites by MTX that will be required to suppress cellular THF levels, and (*d*) the higher the levels of free intracellular MTX that will be required to achieve this endpoint. Conversely, the higher the rate of cellular deoxythymidylate synthesis from deoxyuridylate (*a*) the higher the cellular DHF level, (*b*) the greater the fraction of total enzyme that is required to sustain THF reduction in the absence of MTX, (*c*) the lower the degree of saturation of DHFR sites by MTX that will be required to suppress cellular THF levels, and (*d*) the lower the level of free intracellular MTX that will be required to achieve this endpoint. Thus, S-phase cells in logarithmic growth, utilizing the highest percentage of DHFR potential to meet THF demands, would be highly sensitive to MTX not only because the cell is committed to replication of DNA at this point and is, therefore, inherently more sensitive to this "cycle-active" agent, but because the concentration of free drug within the cell that would be required to suppress cellular THF cofactor levels would be low. It is of particular interest, however, as pointed out above, that even in L1210 leukemia cells in exponential growth, only 5% of total enzyme activity appears to be necessary to meet cellular THF requirements (10). Hence, it should not be surprising that under conditions in which cellular replication is not possible and the rate of DNA synthesis is quite low, as in in vitro studies, the fraction of DHFR necessary to sustain basal rates of THF synthesis would be even smaller and the highest levels of free intracellular MTX would be required to suppress this low rate of THF synthesis.

Sirotnak and Donsbach suggested that when in-

tracellular MTX is at molar equivalence to the DHFR binding capacity in the L1210 leukemia in vivo, ^{3}H-UdR incorporation into DNA is completely inhibited (9). However, the very small levels of exchangeable drug that are present within the cell when the extracellular MTX concentration is low can be very difficult to detect. For instance, these authors reported that the DHFR binding capacity for MTX in the L1210 leukemia is 3.75 nmols/g dry weight. When extracellular MTX is 10^{-8} M, assuming the steady-state exchangeable drug concentration to be the same, this would be equivalent to an intracellular MTX level of 0.0375 nmols/g dry weight or only 1% of the total cellular binding capacity. Indeed, even when the extracellular MTX concentration is 5×10^{-8} M, an equivalent amount of exchangeable drug within the cell would represent only 5% of the total binding capacity. While these levels of exchangeable intracellular MTX are not trivial in that they represent a concentration of drug which is three orders of magnitude above the MTX K_i, they are in a range that would be difficult to distinguish from the component of MTX tightly bound to DHFR. Careful analysis of published data suggests that incorporation of ^{3}H-UdR into DNA does persist in vivo even under conditions in which the level of intracellular MTX is *in excess* of the tightly bound fraction (chart 5, ref 29). Further (chart 13, ref 9), when animals bearing the L1210 leukemia were given MTX at 0.3 mg/kg, incorporation of ^{3}H-UdR into DNA in vivo continued at ~20% of control values even when the exchangeable intracellular MTX level was ~0.13 μM 30 minutes after drug administration, but as the exchangeable intracellular drug level was increased, ^{3}H-UdR incorporation was further suppressed. In fact, there is considerable evidence from in vivo studies which suggests that the level of free extracellular MTX, and, by inference, the free intracellular MTX concentration, determines the degree and duration of inhibition of ^{3}H-UdR incorporation into DNA. Margolis et al (1) noted the rapid recovery of ^{3}H-UdR incorporation into DNA in mouse intestine following administration of MTX. Chabner and Young (2) demonstrated that ^{3}H-UdR incorporation into DNA recovers rapidly in L1210 leukemia cells, mouse bone marrow, and gastrointestinal mucosa along with a concurrent cessation of leukemic cell kill as the extracellular MTX level falls to 10^{-8} M. Indeed, the studies of Sirotnak and Donsbach which correlate the rate of fall of exchangeable intracellular MTX with suppression of ^{3}H-UdR incorporation into DNA (29) and cytotoxicity (8) in vivo imply an important role for free intracellular MTX. Likewise, these authors have suggested the importance of the exchangeable intracellular drug component of other antifols in achieving cytotoxicity (30). Finally, it has been shown that the critical element in the apparent linear relationship between MTX influx in vitro and MTX cytotoxicity to murine leukemias in vivo reported by Kessel et al (31) is not due to differences in influx per se among the different cell lines in terms of the intervals required to saturate DHFR sites within the cell (7,32). Rather, cytotoxicity must be related to differences in the absolute level of exchangeable intracellular MTX achieved (33), the rate of decline of the free intracellular drug concentration following pulse administration in vivo (8), and possibly the inhibitory effects of extracellular MTX on THF cofactor influx into the cell (*see* section entitled Role of Sustained Exposure of Cells to Extracellular MTX in the Suppression of THF-Dependent Processes), parameters of the drug-cell interaction which are controlled by the membrane transport system and parallel the rate of flux of MTX into the cell (7,32).

Consideration of a Possible Inhibitory Effect of MTX on Non-S-Phase Cells in High-Dose Protocols

As a "cycle-active" agent, MTX is considered to achieve its cytotoxic effects by inhibiting DNA synthesis in the S-phase cell (34). This may be based not only on the inherent sensitivity to this agent of S-phase cells committed to replication of DNA, but in addition to the diminished requirement for free intracellular MTX in the suppression of THF synthesis in cells which utilize the highest fraction of their DHFR capacity to meet their THF requirements. However, nonproliferating cells in vitro (3–6), as well as stationary cells in their culture growth medium (35), continue to incorporate ^{3}H-UdR into DNA and hence require THF regeneration albeit at a considerably lower rate than cells in logarithmic growth. DNA synthesis in stationary cells has been attributed to a small fraction of the total cell population in S-phase (35). In addition, this ^{3}H-UdR incorporation into DNA may be due to mitochondrial DNA synthesis or DNA repair in non-S-phase cells. Irrespective of the cell cycle phase, if deoxythymidylate is synthesized from deoxyuridylate, DHF is generated and the maintenance of THF cofactor levels will require regeneration of THF from DHF by DHFR. Inhibition of this process would represent a potential for depletion of cellular THF cofactors and, ultimately, the cessation of THF-dependent processes. The lethal effect of MTX under these conditions in which replication of DNA may not be initially critical for cell survival might be related to inhibition of DNA repair or to the depletion of THF cofactors required for RNA syn-

thesis and the depletion of short-lived proteins. According to the model described above (*see* preceding section), for MTX to suppress DNA synthesis in these cells would require very high concentrations of drug to inhibit the very small fraction of DHFR activity necessary to sustain THF levels. It is of interest that, experimentally, the ability of MTX to suppress ^{3}H-UdR incorporation into DNA and to kill cells in logarithmic phase growth is considerably greater than in cells in stationary phase (35). The possibility that MTX may be toxic to cells out of S phase is further raised by the observation that high-dose MTX protocols inhibit slow-growing solid tumors. While this agent might achieve its effects in these treatment regimens by the destruction of a small fraction of the tumor cells which are in S phase, it is also possible that the agent acts on nonreplicating cells. It should be pointed out, of course, that in the cell which does not synthesize deoxythymidylate from deoxyuridylate at all, there would be no intracellular mechanism by which THF cofactor stores would be depleted and this kind of cell should be completely insensitive to MTX.

The ability of MTX to achieve its biochemical effects in the non-S-phase cell would be determined by a number of factors. (*a*) As indicated above, the highest levels of free intracellular drug would be necessary to inhibit basal rates of THF synthesis. (*b*) Since the THF requirement is very small, submaximal inhibition of THF regeneration within the cell by MTX may lead to only a very slow rate of depletion of THF cofactors so that the interval of exposure to MTX must be very long. (*c*) Under these conditions, high levels of THF cofactor stores within the intracellular compartment could play a role in sustaining cellular THF cofactor levels sufficient to maintain cell viability over the interval of exposure to MTX. The apparent capacity of MTX to inhibit very slow-growing tumors as well as these theoretic considerations warrant further exploration of the pharmacologic effects of MTX in the non-S-phase cell.

Pharmacologic Endpoints for MTX: Free Intracellular MTX Levels Achieved *During* Drug Administration Versus the Rate of Decline of Free Intracellular MTX *Following* Drug Administration

Membrane transport properties for MTX can affect a number of elements of the drug-cell interaction which could impair MTX cytotoxicity (32). (*a*) A low unidirectional flux of MTX into the cell (influx) will increase the interval required to achieve saturation of DHFR sites since influx is rate limiting to binding within the cell. A low influx on this basis may limit cytotoxicity when the interval of exposure to drug is short in comparison to the duration of time required to saturate high-affinity binding sites at a given extracellular drug level. This was considered in detail in reference 32. (*b*) A low influx may be associated with a reduction in the steady-state level of free drug achieved, and (*c*) an increased rate of decline of exchangeable intracellular drug as the extracellular drug concentration falls following cessation of drug administration. (*d*) A high rate of unidirectional drug efflux may reduce the steady-state level of free drug achieved within the intracellular water and increase the net rate of decline of exchangeable intracellular drug as the extracellular drug concentration falls following cessation of drug administration (32). It is obvious that a *critical* level of free intracellular MTX must be achieved in a given drug regimen to effect cessation of THF synthesis and that this intracellular drug level must be sustained for a critical interval to effect cytotoxicity. After a low-dose pulse of MTX, critical levels of free drug may be sustained within the cell only transiently so that the net rate of decline of the free intracellular drug concentration as the extracellular drug level falls may be the important factor which determines the interval over which free intracellular MTX is in excess of the critical level (8,29). In high-dose MTX "rescue" protocols, however, high drug levels are sustained over long intervals either by a continuous infusion of drug or by high-dose pulses, followed by "rescue" with 5-formyl-THF. Hence, the interval of exposure to drug is long and the metabolic block induced by MTX presumably ceases with "rescue" at a time when free intracellular MTX is in excess of the critical level. In these regimens, the critical pharmacologic endpoint for MTX may be the absolute level of drug achieved and the interval of drug administration. The rate of fall of free intracellular MTX following cessation of administration of MTX and initiation of the "rescue" with pharmacologic doses of 5-formyl-THF is not likely to be an important factor unless delivery of THF cofactors to the intracellular compartment is required in excess of substrate quantities due to, for instance, MTX and THF cofactor competition for enzyme(s) within the cell. While competition between MTX and 5,10-methylene-THF for thymidylate synthetase can be observed in vitro, this is probably not an important consideration during the "rescue" because of the relatively low concentrations of free MTX which accumulate within the cell and the low affinity of thymidylate synthetase for this agent (19,36). This will, however, require further study. Hence, the critical elements for the MTX-cell interaction which influence the pharmacologic efficacy of this agent will depend to a large

extent on the specific protocols employed and the cell types involved.

Role of Sustained Exposure of Cells to *Extracellular* MTX in the Suppression of THF-Dependent Processes

Experiments which confirmed the critical role of free intracellular MTX in the suppression of THF-dependent processes were designed to exclude interactions between extracellular MTX and THF cofactors at the level of their common membrane carrier (3–6). This required in vitro studies in which THF cofactors could be eliminated from the extracellular compartment. Indeed, the critical role for free intracellular MTX in in vivo studies in the inhibition of THF-dependent processes must be extrapolated from in vitro experiments which ensured that extracellular MTX does not alter transport of THF cofactors into or out of the cell, leaving intracellular drug as the critical factor. This is a conclusion now supported by the observation that free drug is required to inhibit THF synthesis, an intracellular event (6). However, it is important to point out that although a major requirement for sustained exposure of cells to extracellular MTX to achieve cytotoxicity with this agent in vivo may be attributed to the role of free intracellular drug, extracellular drug may have effects in addition to the maintenance of free intracellular drug. Hence, under conditions in which there is sufficient free intracellular MTX to suppress THF synthesis, extracellular MTX may inhibit the transport of extracellular THF cofactors into the intracellular compartment (37) which might sustain THF-dependent processes during the interval over which the cell is deprived of endogenous THF regeneration. Likewise, the rate of decline of extracellular MTX may be an important factor in 5-formyl-THF "rescue" by inhibiting influx of THF cofactors in substrate quantities following cessation of MTX administration and initiation of the "rescue" (38). Hence, the efficacy of high-dose regimens may be related not only to the generation of free MTX within the cell with the cessation of THF synthesis but, in addition, to the concurrent inhibition of THF cofactor delivery into the cell by extracellular drug.

Limited Capacity of Mammalian Cells to Accumulate Free Intracellular MTX: Possible Role for Microtubular Inhibitors in High-Dose MTX Protocols

Early studies from this laboratory suggested that the exchangeable intracellular MTX level is a saturable function of the extracellular drug concentration in the L1210 leukemia approaching a maximum of ~6.5 μM as the extracellular MTX is raised to levels which saturate the carrier (11). A similar qualitative relationship has been observed in the L-cell mouse fibroblast and the Ehrlich ascites tumor (3). We have recently reported that this relationship appears to be valid, experimentally, even at MTX levels comparable to those employed in high-dose protocols (12). Figure 2 illustrates uptake of exchangeable MTX in Ehrlich ascites tumor cells over 1 hour at extracellular drug concentrations of 10, 100, and 250 μM in the presence or absence of 10 μM vincristine; accumulation of exchangeable drug is markedly restricted. At a concentration of 100 μM, the steady-state exchangeable drug level is ~5 μM, about one twentieth the extracellular level. Even when the extracellular MTX concentration is 250 μM, exchangeable intracellular drug does not exceed 10 μM over a 60-minute interval of incubation. MTX accumulation is quite limited in Sarcoma 180 cells (39), human leukocytes, and leukemia cells as well (40–42). The basis for this restricted accumulation of MTX in mammalian cells is not entirely clear. Hakala (39) and this laboratory (37,43) suggested that an energy-dependent process pumps MTX out of the cell. This is supported by the observation that metabolic poisons augment the net accumulation of MTX in a variety of mammalian cells (37,39,43). The very low steady-state distribution ratio for MTX (the ratio of the concentration of exchangeable intracellular to extracellular drug) further suggests an energy-dependent exit mechanism. However, since MTX is a bivalent anion, it would be expected even for a passive transport system that the distribution ratio for MTX would be some value less than one, dependent upon the membrane potential. In the Ehrlich ascites tumor, for example, the membrane potential may be at least -24 ± 7 mv (45); this alone would account for a distribution ratio of 0.15. The significance of chemical gradients for MTX across mammalian cell membrane will be better understood when membrane potentials can be measured accurately under experimental conditions comparable to those in which the MTX distribution ratio is generated. Finally, exclusion of MTX from aqueous intracellular compartments due to membrane permeability barriers or localized pH or electrical gradients across membranes surrounding intracellular compartments might further contribute to "apparent" low distribution ratios for this agent.

Vincristine and other vinca alkaloids augment net accumulation of intracellular MTX over a broad range of extracellular drug concentrations (fig 2). This is associated with enhanced inhibition by MTX of ^{3}H-UdR incorporation into DNA in vitro (4) and an augmentation of MTX cytotoxicity to the L1210

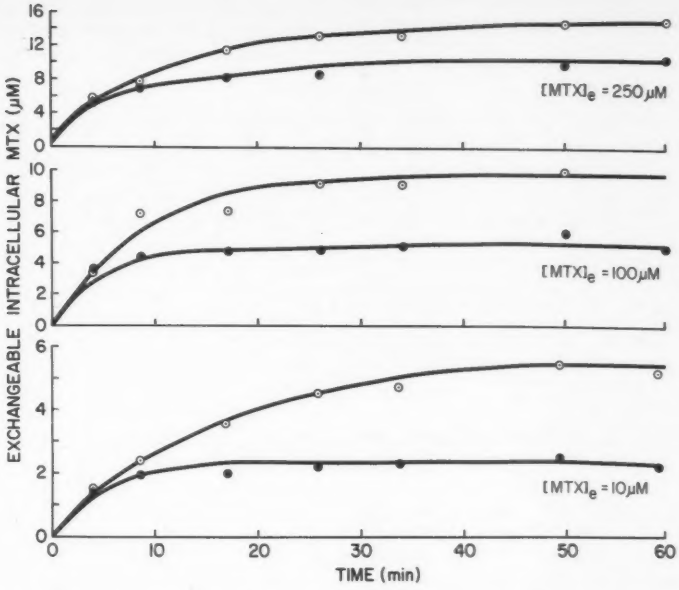

FIGURE 2.—Time-course of uptake of exchangeable intracellular MTX at extracellular MTX concentrations of 10, 100, and 250 μM in the presence (⊙) or absence (●) of 10 μM vincristine. Exchangeable intracellular MTX levels were determined from the differences between total cell MTX and MTX that remains in the Ehrlich ascites tumor cells after resuspension into MTX-free buffer. (*From* ref 12.)

leukemia in vivo (46). We have proposed that vincristine may act in this context as an inhibitor of energy metabolism blocking the energy-dependent efflux of MTX (44). The augmentation by vincristine of net uptake of MTX may represent, at least in part, a biochemical basis for the apparent synergism between these agents and a rationale for their combined use in clinical regimens. It should be pointed out, however, that it is not as yet clear whether vincristine levels achieved in clinical protocols are sufficiently great to be of importance in the treatment of human tumors. While the concentration of vincristine required to enhance net MTX transport into animal tumors is in excess of 10^{-6} M (44,46), Bender et al (41) have reported that 10^{-7} M vincristine produced a 30% increase in net MTX accumulation in human leukemia cells, which would represent an even larger increase in the free intracellular component. Further, it is unclear as to whether the vincristine effect is selective, ie, that the augmentation of MTX accumulation in tumor cells is more pronounced than in susceptible host tissues. Finally, since vinca alkaloids are rapidly cleared from the blood (47), vincristine effects on MTX accumulation in vivo might be quite transient since in vitro augmentation of net MTX accumulation by vincristine is partially reversible after the agent is removed from the extracellular compartment (44). Should further studies support the use of this agent in the treatment of human malignancies, it may be a potentially valuable adjunct to high-dose MTX therapy. Since nephrotoxicity may limit the MTX dose due possibly to the deposition of drug in the renal interstitium and tubules (48), vincristine provides an opportunity to augment the net intracellular MTX level without a change in the extracellular drug concentration. This is a particularly important consideration since accumulation of free intracellular MTX is very restricted in mammalian cells, and at high extracellular MTX concentrations, to achieve small increases in the free intracellular MTX level requires very large increases in the MTX dose (fig 2). This has been analyzed recently (12).

Roles of MTX K_i, Cellular DHFR Levels, and Cellular THF Cofactor Pool Size as Determinants of Sensitivity to MTX

Jackson and Harrap have explored this issue extensively (10) and their findings as well as other considerations are reviewed here. Since the interac-

tion between MTX and DHFR is competitive with DHF, as DHF within the cell rises from trivial levels to concentrations which permit competition with MTX for a small percentage of total DHFR, the cellular DHFR level within the cell is a critical factor in determining cellular sensitivity to MTX. It should be pointed out, however, that although the total DHFR level of the cell may go up by a large factor when cells become resistant to MTX, it is only a small percentage of the total enzyme increase which permits THF synthesis to proceed when the major portion of the enzyme is inhibited by MTX. Hence, if only 2% of the enzyme is necessary to sustain cellular THF levels, if the total enzyme level doubles, only 1% of the enzyme is now sufficient to meet the cellular THF requirement and inhibition of this smaller enzyme fraction will require much higher levels of free MTX within the cell. Similarly, since cessation of THF synthesis is achieved by *competition* between MTX and DHF, an increase in the MTX K_i or a decrease in the DHF K_m may profoundly affect the degree of suppression of THF synthesis at a given free intracellular MTX level. For instance, increase in the MTX K_i from 10^{-11} to 10^{-10}, while still representing a high-affinity binding of MTX, would nonetheless appreciably diminish the effectiveness of this agent.

Finally, Jackson and Harrap evaluated the effects of an increase in the THF cofactor pool on cellular sensitivity to MTX. They predicted that a sevenfold increase in the pool size will delay the effect of 10^{-7} M MTX on log phase cells by only 20 minutes with only a small (~22%) increase in the LD50. Indeed, even in in vitro systems, ^{3}H-UdR incorporation into DNA ceases a few minutes after exposure to concentrations of MTX sufficient to abolish THF synthesis (3) so that THF cofactor pools must be trivial under these conditions and even a large increase in their size would negligibly affect the interval over which THF-dependent processes within the cell are inhibited. However, under conditions in which the basal rate of cellular DHF synthesis is very low, and the cellular MTX levels achieved produce only a small reduction in THF synthesis, the cellular THF cofactor pool might be sufficiently great to sustain the cells through some finite interval of exposure to MTX. It should be pointed out also that the influence of THF cofactor pool size on MTX effects is based not only on their role as substrate for THF-dependent processes when cellular THF synthesis is blocked. In addition, the THF cofactor level in the cell will determine the DHF level that can be achieved when THF cofactors revert to the oxidized form. This determines the level of DHF which will be available for competition with MTX for DHFR sites.

Differential Effect of MTX on Thymidylate Versus Purine Biosynthesis: Rationale for Thymidine "Rescue"

Studies from this laboratory have demonstrated that the level of exchangeable MTX within the cell determines the degree to which purine biosynthesis is inhibited relative to inhibition of thymidylate synthesis (fig 3). Hence, ~0.3 μM exchangeable intracellular MTX produced a 50% inhibition of ^{3}H-UdR or ^{14}C-formate incorporation into DNA but an exchangeable intracellular MTX level of ~1 μM was required to produce comparable inhibition of ^{14}C-formate incorporation into RNA (5). This may be related to alterations in cellular coenzyme levels due to the properties and quantities of interconversion enzymes affecting individual cofactors differently as the total THF level falls as well as the relationship between specific cofactor levels and their K_m. These observations suggested that at low extracellular and intracellular MTX concentrations, the primary effect of MTX may be related to inhibition of thymidylate synthesis, while at higher MTX concentrations, both biosynthetic processes should be inhibited. Hryniuk has shown that it is the inhibition of purine biosynthesis by MTX which is the critical factor in the initial drug-induced cytotoxicity and suppression of DNA synthesis in L5178Y leukemia cells (49,50). This was attributed to the inhibition by MTX of the synthesis of deoxypurine nucleotides required for DNA synthesis. Studies by Grindey and Moran further support this view (51). These considerations may clarify one basis for thymidine "rescue": Tattersall et al (52) observed that thymidine rescues L1210 leukemia-bearing mice from the toxic effects of MTX but does not minimize MTX cytotoxicity to the tumor cells. Likewise, Semon and Grindey report that when MTX is infused in the tail vein of mice bearing the L1210 leukemia to achieve low extracellular drug levels, concurrent infusion of thymidine decreases toxicity to the host while maintaining toxicity to the tumor. However, at high MTX blood levels, both inosine and thymidine are required to rescue the host but this results in tumor rescue as well (53). If the level of free MTX achieved in susceptible host cells is below that in the tumor cell, the major effect in the former may be the suppression of thymidylate synthesis while the major effect in the latter will be the combined block of both purine and thymidylate synthesis. Under these conditions, thymidine rescues the host cell which is "at risk" due to a block in thymidylate biosynthesis. While the thymidylate block in the L1210 cell is also reversed, the purine block persists and results in cell death. This formulation is supported by the observation that ex-

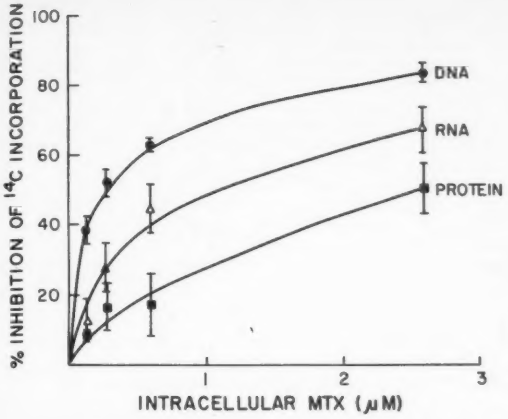

FIGURE 3.—Percentage of inhibition of ^{14}C-formate incorporation into RNA, DNA, and protein by exchangeable intracellular MTX in L-cell mouse fibroblasts. (*From* ref 5.)

changeable MTX rapidly leaves the mouse intestinal cells but is sustained considerably longer in the L1210 leukemia following pulse administration of the drug in vivo (29). These considerations raise the possibility that thymidine "rescue" may have particular application to the treatment of tumors of the central nervous system, especially disease involving the meninges. Direct infusion of MTX into the CSF may produce sufficiently high MTX levels to block both purine and pyrimidine synthesis. However, concurrent MTX blood levels may be sufficiently low to result in inhibition of thymidylate synthesis alone in susceptible marrow and gastrointestinal epithelial cells which can be reversed with a systemic thymidine infusion.

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Lipid-Soluble Diaminopyrimidine Inhibitors of Dihydrofolate Reductase

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SUMMARY

On the basis of activity against experimental tumors and potency as inhibitors of human dihydrofolate reductase, two compounds were selected for pharmacokinetic evaluation: metoprine ((2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine, DDMP, B.W. 197U) and etoprine, the corresponding 6-ethyl analog (DDEP, B.W. 276U). These lipid-soluble compounds readily cross the blood-brain barrier and penetrate rapidly into brain and brain tumors induced in rats by ethylnitrosourea. Both compounds are extensively bound to human plasma protein and their slow elimination from plasma and tissues contrasts with the kinetics of methotrexate. Cerebrospinal fluid levels of "folate" were elevated following oral administration of citrovorum factor to rats but not following equivalent doses of folic acid. The balance between selective action of the drug and selective protection by the vitamin is discussed with regard to differential distribution into separate compartments.

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Despite some uncertainty about the integrity of the blood-brain barrier in the area of a brain tumor, a degree of lipid solubility appears to be an essential property of those drugs which remain as candidates for the chemotherapy of brain tumors (1,2). The antimalarial drug, pyrimethamine (Daraprim) (3), known to penetrate the cerebrospinal fluid (CSF) from its usefulness in the treatment of toxoplasmosis, induced remissions of > 6 months' duration in the treatment of meningeal leukemia (4). During the early evaluation of phenylpyrimidines related to pyrimethamine, Burchenal and co-workers (5) reported two observations in 1952 which have been borne out by subsequent studies: (*a*) the 3′,4′-dichlorophenyl compounds were more potent anticancer agents than the 4′-monochlorophenyl compounds, and (*b*) these dichlorophenyl pyrimidines were active against methotrexate (MTX)-resistant cells (AK_4 leukemia). Subsequent laboratory and clinical studies of metoprine (2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine, DDMP) used daily administration of this drug since analytic methods to define kinetic parameters had not been developed. In retrospect, it is not surprising that toxicity occurred in patients treated daily with DDMP (6), resulting in this compound being set aside, since drug accumulation to toxic levels can now be related to its very long persistence in plasma and tissues.

Resurgence of interest in DDMP and etoprine (DDEP), the corresponding 6-ethyl analog, is related to recognition of their activity against MTX-resistant tumors and to their rapid entry into body compartments (brain, pancreas, testes) from which more polar anticancer drugs are excluded. Treatment with these drugs, combined with protection of bone marrow and mucosal tissues by citrovorum factor (CF), seems to afford the prospect of extending therapy with folic acid antagonists to solid tumors unresponsive to MTX. The purpose of this paper is to present data on the physical properties, plasma protein binding, and kinetic parameters of DDMP and DDEP and to relate the kinetics of the vitamin to protection of normal tissues while avoiding "rescue" of malignant cells.

MATERIALS AND METHODS

Pyrimethamine, DDMP, and DDEP labeled with ^{14}C in position 2 of the pyrimidine ring (13.8, 53.7, and 14.7 millicuries/mM, respectively) were used for kinetic determinations in the rat. Unlabeled drug

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[2]We wish to thank Mr. Aris Ragouzeous for determining the octanol-water partition coefficients in table 1.

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TABLE 1.—Comparative lipid solubility, protein binding, and dihydrofolate reductase inhibition of 2,4-diaminopyrimidines and MTX

	Physical properties		Binding to human plasma protein (%)	Inhibition of human dihydrofolate reductase†: concentration for 50% inhibition (M × 10^8)	
	pKa	log P*		Erythrocyte enzyme	ALL enzyme
Pyrimethamine	7.34	2.69	87§	21	26
DDMP	7.15	2.82	88§	6	5
DDEP	7.20	3.19	97§	1	1
MTX	4.7‡	−1.85‡	53¶	0.1	0.1

*Octanol-water partition coefficient determined by the method of Leo et al (ref 25).

†Each enzyme preparation was purified by affinity chromatography from human erythrocytes and from acute lymphocytic leukemic (ALL) cells from infiltrated human spleen. The assays were done at pH 7.5 with 100 μmols of Tris buffer, 150 μmols of KCl, 0.05 μmol of NADPH, and 0.02 μmol of dihydrofolate/ml (Bertino JR, personal communication).

‡pKa determined in 30% methanol water (Hansch C, personal communication).

§Binding to human plasma protein determined by equilibrium dialysis at pH 7.4 with a concentration of 1 μg/ml.

¶Binding to serum protein determined by ultrafiltration at a concentration of 10 μg/ml (ref 26).

was used for studies in the dog and in man. Folic acid was supplied by Sigma Chemical Co (St. Louis, Mo) and calcium leucovorin (*dl*-5-formyl tetrahydrofolate, CF) by Lederle Laboratories Division of American Cyanamid Co (Pearl River, NY). The analytic procedures used for each experiment are described in the tables and figures.

PHYSICAL CHARACTERISTICS AND BIOLOGIC PROPERTIES OF THREE 2,4-DIAMINOPYRIMIDINES

Based upon their octanol-water partition coefficients (P), these folate antagonists are among the most lipid-soluble anticancer compounds. The lipid solubility of DDMP is close to that of CCNU and the lipid solubility of DDEP is comparable to that of methyl-CCNU. This is in striking contrast to the lack of lipid solubility of MTX which, as a polar compound, has a negative log P. Also, the dissociation constants indicate that these compounds are ~50% ionized within the physiologic pH range (table 1). Their rapid penetration across the blood-brain barrier and distribution into different body compartments are undoubtedly related to this lipophilic property. It is likely that these compounds are bound to lipids or lipoproteins in tissues or to the phospholipids of membranes as storage depots from which they are gradually released into the circulation. Their extensive binding to plasma protein may also contribute to the long persistence of the compounds in blood if the rate of metabolism is slow. The data shown in table 1 indicate that DDEP is bound more than the other two compounds. Such high affinity for plasma proteins could be a disadvantage in restricting the proportion of free drug. Also, there is potential for adverse interactions with other drugs which might cause displacement of the bound cytotoxic agent. This extensive binding of the diaminopyrimidines to plasma proteins also contrasts with the much lower binding of MTX.

These diaminopyrimidines are known to be potent inhibitors of dihydrofolate reductase and their activity in this regard has been studied using enzyme preparations from microbial and mammalian cells (7,8). The information most relevant to their clinical use, however, is their comparative potency against human dihydrofolate reductase. We are indebted to Dr. J. R. Bertino (Yale University, New Haven, Conn) for the comparison shown in table 1, using dihydrofolate reductase preparations purified by affinity chromatography from human erythrocytes and from acute lymphocytic leukemic cells from infiltrated human spleen. On the basis of the concentration required for 50% inhibition, both DDMP and DDEP are considerably more potent than pyrimethamine. DDEP approaches the effective concentration of MTX, being about one tenth as active. Because of their lower affinity for dihydrofolate reductase, these compounds do not have the same stoichiometric pattern of inhibition as MTX and consequently would be expected to be less effective in the presence of high concentrations of dihydrofolate.

PERSISTENCE OF DIAMINOPYRIMIDINES IN PLASMA FOLLOWING ORAL ADMINISTRATION

The prolonged persistence of pyrimethamine in body fluids was recognized from early studies showing the long duration of its action in the prophylaxis of malaria. A plasma half-life of 100 hours was calculated from the plasma levels assayed microbiologically by Smith and Ihrig (9). This was confirmed using a direct assay based on quantitative thin-layer chromatography in our laboratory and found to average 92 hours in healthy human subjects (10).

TABLE 2.—Species comparison of plasma half-life data for 3 diaminopyrimidines*

	Plasma half-life (hrs)		
	Rat	Dog	Man
Pyrimethamine	9	35	92
DDMP	18	57	180
DDEP	12	36	Unknown

*Male Sprague-Dawley rats received the radiolabeled 2,4-diaminopyrimidines as an oral dose (3 mg/kg) in a 0.01 N lactic acid solution. Animals were killed in groups of 3 at various time intervals and blood and CSF were sampled. Total radioactivity was determined in plasma and CSF by counting in Aquasol-2, NEN by liquid scintillation counting using a Packard instrument model 3320 and corrected for quench by internal spiking. For the dog and human studies, intact DDMP and DDEP were determined by a quantitative thin-layer chromatographic (TLC) method (ref 27). Male beagles (~10 kg) were fasted before receiving drug orally at a dose of 10 mg/kg. Fasted adult men received 100 mg pyrimethamine orally and in a second study at a later time received 50 mg DDMP orally (ref 10).

TABLE 3.—Drug distribution 5 hrs after oral doses of DDMP and DDEP*

	DDMP (μg/g)	DDEP (μg/g)
Plasma	0.98 ± 0.23	1.11 ± 0.20
Lung	25.4 ± 8.1	15.4 ± 8.4
Brain tissue	7.5 ± 1.3	5.4 ± 1.5
Brain tumor	10.0 ± 4.2	4.3 ± 2.4

*Rats bearing ethylnitrosourea-induced brain tumors received 10 mg/kg of each drug orally; extraction procedure and assay by quantitative TLC are described in ref 27.

In a similar study, the plasma half-life of DDMP was found to average 180 hours; comparison with DDEP awaits FDA clearance for human studies. However, the half-life data for different species shown in table 2 indicate that DDEP has a half-life in dogs in the same range as pyrimethamine, whereas DDMP persists for a much longer period. The same relationship was observed in studies in rats. The very long half-life of DDMP in man requires prolonged administration of CF to control adverse effects on bone marrow and mucosal tissues. If DDEP has a much shorter half-life than DDMP, then easier control of drug levels in body fluids could be attained by dosage scheduling. Since DDEP is about five times more potent than DDMP as an inhibitor of dihydrofolate reductase, even lower amounts distributed into different tissues could still achieve the same cytotoxic effect as with DDMP.

A more detailed comparison of the elimination of DDMP and DDEP from the plasma of rats given an oral dose of each compound is shown in figure 1. Total radioactivity was measured over a period of several days following a single oral dose of each compound. Although DDEP is more lipid soluble than DDMP, its rate of elimination is considerably more rapid. Of particular interest is the parallel nature of the curves for each drug in the plasma and CSF. Data from other studies measuring the disappearance of each drug from tissues and organs also show the same parallel relationship to the amount present in plasma. The amount present in CSF is consistently lower than that in plasma and may be related to the lower protein content of CSF.

TISSUE DISTRIBUTION AND UPTAKE INTO BRAIN, CSF, AND BRAIN TUMORS

The induction of brain tumors in rats by treatment with ethylnitrosourea has been developed as a model with relevance to the type of tumors occurring in man (11). As part of a collaborative study, we have measured the distribution of DDMP and DDEP in rats bearing such tumors (12). Primary neurogenic tumors were induced in CDF Fischer rats by iv injection with 50 mg/kg of ethylnitrosourea in pregnant dams on Day 20. The offspring were selected when the presence of brain tumors was assured after a period of 8 or 9 months. The data shown in table 3 indicate that a few hours following oral administration of either drug, the amount present in normal brain tissue and in brain tumors was several-fold higher than that present in plasma. In each case, the amounts of DDMP were higher than the levels of DDEP. Also, the substantial concentration of each drug in lung tissue is of interest in relation to the potential usefulness of such agents for treatment of bronchogenic carcinoma. Denlinger et al have observed that treatment with DDMP or DDEP prolongs the survival of rats bearing the induced brain tumors (12).

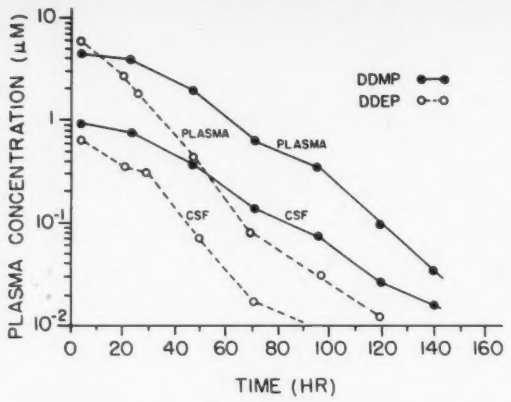

FIGURE 1.—Elimination of radioactivity from plasma and CSF following an oral dose of ^{14}C-DDMP or ^{14}C-DDEP to the rat. For experimental details *see* table 2.

CSF PENETRATION OF "FOLATE" FOLLOWING ORAL DOSES OF FOLIC ACID OR CF

An ideal circumstance for selective treatment of brain tumors would be to combine a drug which enters rapidly into CSF and brain with a protective agent which could act systemically without penetration of the blood-brain barrier. BCNU and CCNU meet the first qualification but their combination with a systemic neutralizing agent has not yet been attempted. DDMP and DDEP penetrate readily into brain tumors in experimental studies (table 3) and may have some selective action against brain neoplasms since brain tissue lacks dihydrofolate reductase (13). CF can provide systemic protection against the toxic effects of DDMP and DDEP but the extent and rate of penetration of 5-methyltetrahydrofolate (5-methyl-THF) into CSF and brain may be related to dosage. Experimental studies have shown that 5-methyl-THF is preferentially taken up into CSF (14) and its entry into the central nervous system (CNS) via the choroid plexus in rabbits is a saturable process (15,16). The differential distribution of drug and vitamin into each compartment requires careful study.

A comparison of the plasma and CSF levels of "folate" following oral administration of folic acid or CF to rats is shown in figure 2. "Folate" was determined by a radioassay technique using β-lactoglobulin as a competitive binding agent (17). The actual amounts measured are less important than comparison of the patterns observed. Both plasma and CSF levels of 5-methyl-THF were elevated following CF treatment. In contrast, no increase in CSF levels occurred following administration of folic

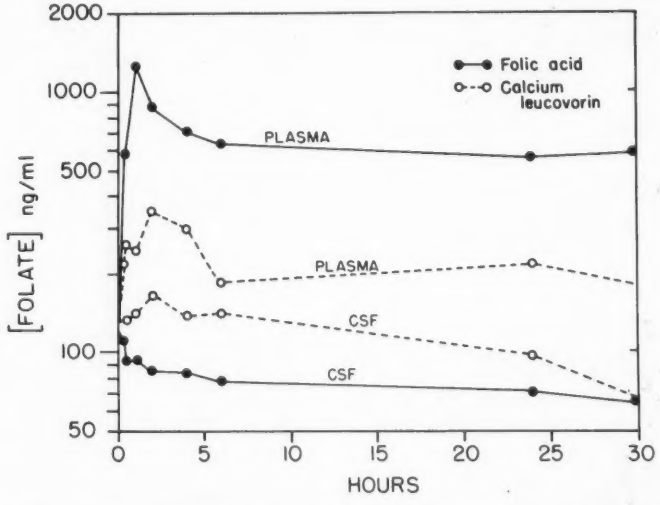

FIGURE 2.—Elimination of "folate" from plasma and CSF following a single oral dose of folic acid (6 mg/kg) or CF (6 mg/kg) to the rat. For "folate" kinetic studies 27 male Sprague-Dawley rats/group were fasted overnight and throughout the experiment. Just prior to dosing, 3 rats were killed and plasma and CSF removed for 0 time controls. 24 rats were dosed with CF (6 mg/kg, orally) as a water solution and 24 rats were dosed with folic acid (6 mg/kg, orally) as a 0.5% sodium lactate solution. Animals were killed in groups of 3 at various time intervals. Folate in plasma and CSF was assayed by the method of Dunn and Foster (ref 17) which uses β-lactoglobulin as a competitive binding agent. The standard used for these determinations was 5-*N*-methyl-THF.

acid. An adequate explanation for this different pattern is lacking since one would expect prompt conversion of either form of the vitamin to 5-methyl-THF in vivo. This observation has some similarity to the reports that patients receiving anticonvulsants for long periods had lowered levels of CSF folate which were not restored to normal by the administration of folic acid (18). The evidence from this experiment, however, does caution against the use of large doses of CF which might impair the chemotherapeutic response of neoplasms within the CNS to treatment with DDMP.

COMPARTMENTAL CHEMOTHERAPY

The use of CF following high-dose MTX is a type of sequential chemotherapy in which the protective agent must be given in time to "rescue" stem cells in bone marrow and crypt cells in the intestinal epithelium after the target neoplastic cells have been, hopefully, irreversibly damaged (19). DDMP is similar to MTX with regard to the same drug receptor (dihydrofolate reductase) and the same tissues of limiting toxicity (bone marrow and mucosal tissues). The very long persistence of DDMP in plasma and tissues, however, precludes any high-dose "pulse-administration" such as is used with MTX and requires the presence of the protective agent for relatively long periods. Thus, in this case, we are forced to consider the concept of simultaneous administration of drug and protective agent during the course of treatment. Differences in transport systems and rates of uptake into cell or tissue compartments are undoubtedly related to the selective action of drugs and nutrients on particular tissues. While being reluctant to coin another term, there is some advantage to emphasizing pharmacokinetic considerations by using the term "compartmental chemotherapy." Specific biochemical and physiologic characteristics which make cells and tissues different from one another can account for some selective action of folate antagonists and some selective protection by CF. Thus, the concept of "compartmental chemotherapy" visualizes two separate compartments involving (*a*) some selective action of the drug on target cells and (*b*) some selective protection for tissues of limiting toxicity. Clearly, if the protective agent cancels out the drug action on malignant cells, then nothing has been gained. If there is limited entry of the protective agent into the target compartment (CSF, brain tumors, or MTX-refractory neoplasms), then there is the real possibility of amplifying the chemotherapeutic action. That this is feasible has been demonstrated by experiments using a Sarcoma 180 model. The chemotherapeutic index of DDMP was improved by intermittent dosage with drug followed by treatment with CF (20). Also, studies comparing the Murphy-Sturm lymphosarcoma and Walker 256 carcinosarcoma have shown differences between these two tumors with regard to (*a*) response to a dietary deficiency of folic acid, (*b*) uptake of radioactive folate, and (*c*) sensitivity to MTX and DDMP (21). Changes in the transport characteristics of cells in culture were associated with MTX resistance and degree of protection by CF (22).

A pharmacokinetic evaluation involving both drug and protective agent is more complex than the usual phase I clinical study. In the clinical evaluation of DDMP combined with CF treatments, there is already evidence for the protective action of CF (23,24). There is also evidence from monitoring CSF levels of "folate" in patients receiving relatively small doses of CF that the vitamin does penetrate the blood-brain barrier (24). Thus, careful monitoring of drug and vitamin in body fluids is necessary to provide dosage recommendations for both agents. The early clinical reports do indicate some activity of DDMP against neoplasms resistant to MTX and several solid neoplasms known to be refractory to drug treatment (23,24). Despite the long duration of antifolate action, the toxicity of DDMP appears to be controllable. There is reason to expect that the use of such lipid-soluble diaminopyrimidines may extend the spectrum of malignancies responsive to folate antagonists.

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High-Dose Methotrexate Therapy With Citrovorum Factor: A Pharmacologic Perspective in Murine Tumor Models [1,2]

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SUMMARY

Methotrexate (MTX) uptake in tumor cells and mouse small intestine after sc doses up to 800 mg/kg showed saturation kinetics, indicating that carrier mediation and not passive diffusion accounts for entry at high dosages. Maximum accumulation of MTX is much higher in the small intestine, but persistence is much longer in tumor cells. Differences in persistence in tumor versus gut diminish with increased dosage. The duration of inhibition of DNA synthesis, extent of tumor cell kill, and lethal toxicity at different dosages correlate with the retention time for exchangeable drug. The retention of exchangeable drug in tumor and small intestine is reduced by citrovorum factor (CF). Dose dependence for CF-induced drug loss in both tumor and gut is similar, but decreases as the time interval between MTX and CF doses is increased. Little recovery of DNA synthesis in tumor and small intestine was initiated at the time CF was given. Synchronous recovery occurred as drug levels fell to the equivalence of the dihydrofolate reductase content. Earlier recovery of DNA synthesis after CF is a consequence of more rapid drug loss. Although the effect of delayed CF (16 hours) on drug levels is the same in tumor cells and small intestine, much more CF is required to initiate recovery of DNA synthesis in tumor cells. A schedule employing 24 mg/kg of CF sc, 16 hours after 400 mg/kg of MTX sc, gave a two-log tumor cell kill and prevented lethal toxicity in most animals.

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Improved therapy with aminopterin or methotrexate (MTX) when used at normally toxic dosages with citrovorum factor (CF) was shown some years earlier (1,2) and more recently (3,4) in the case of murine leukemia. The effectiveness of similar regimens of therapy has also been shown (*see* refs 5–7 for reviews) in the case of human cancer. Knowledge of specific pharmacologic events which occur following the administration of very high doses of MTX would appear important to the continuing development of this modality of therapy. Moreover, the basis of the selective rescue effect of CF observed either in animal models (1–4) or clinically (reviewed in refs 5–7) is not understood. Mouse studies from other investigators (8) and from our own laboratory (10) associate limiting toxicity of folate analogs to biochemical effects at host renewal site in small intestine. This appears related (10,11) to the duration of inhibition of DNA synthesis, an expression of exchangeable drug persistence (12,13) in tissue.

This paper reviews some recent studies from our laboratory involving tissue pharmacokinetics and biochemical effects of MTX in the L1210 and, to a lesser extent, S180 murine tumor models which are

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[2] Methotrexate: NSC-740, CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-. Obtained from Dr. Harry B. Wood, Drug Development Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI.

Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt. Supplied by Lederle Laboratories, Division of American Cyanamid Co, Pearl River, NY.

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of some relevance to high-dose therapy. We also discuss data showing the effects of CF in these models.

MATERIALS AND METHODS

Details of the experimental methodology have already been described (10,12,13). Beginning 4 days after 10^6 cells were given ip, tumor cells were harvested from the peritoneal cavity in cold buffered isotonic saline (12,13) and drug was extracted by heating (12) after cells were washed. The small intestine was removed, opened longitudinally and segmented, washed, and held in cold buffered isotonic saline for 5 minutes. The tissue was washed again and then homogenized in the same buffered saline (12,13) and drug was also extracted by heating. Drug content in tissue was determined by enzyme assay (12,13) and the dihydrofolate reductase binding content of tissue by titration with MTX (12,13). Tumor cell number in ascites fluid was determined microscopically and measurements of DNA synthesis (10–13) were determined by ^{3}H-deoxyuridine (^{3}H-UdR) incorporation.

RESULTS

Accumulation and Retention of MTX in Tissue

Following administration, the accumulation of MTX in small intestine (fig 1) and L1210 cells (fig 2) is maximum by 2 hours. In L1210 cells, the level of accumulation increased with dosage until 96 mg/kg. In small intestine, accumulation exhibited dose de-

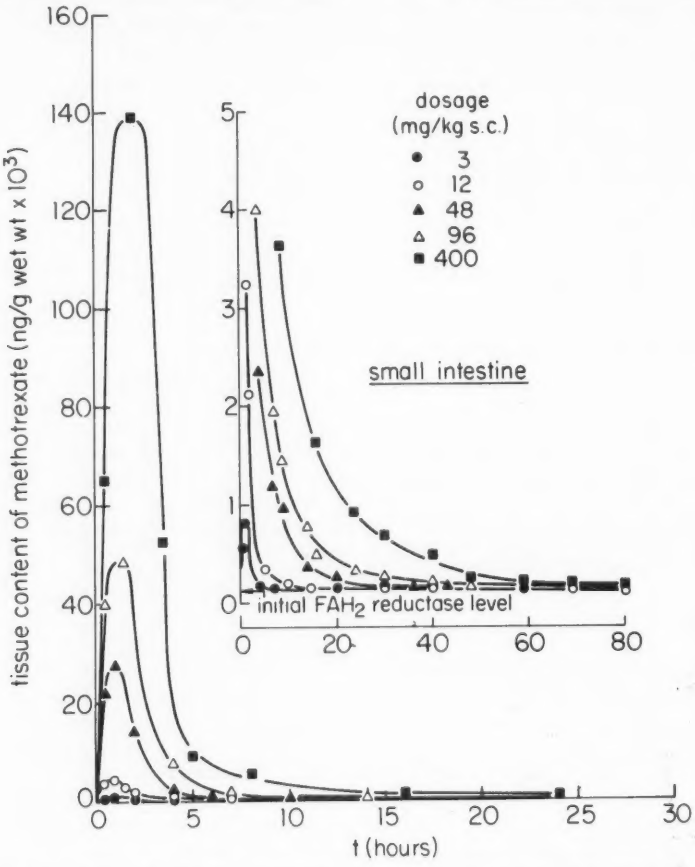

FIGURE 1.—Accumulation and retention of MTX in mouse small intestine after varying sc doses. Values are averages of 2–4 determinations at indicated times obtained in 2–8 experiments. Standard deviation (SD) did not exceed ± 20% of each mean value. Dihydrofolate (FAH_2).

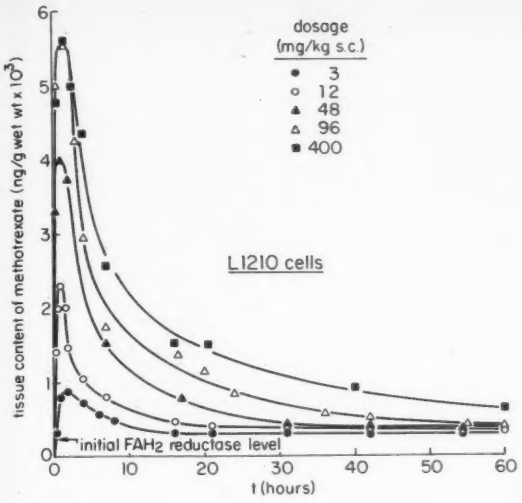

FIGURE 2.—Accumulation and retention of MTX in L1210 cells after varying sc doses. Values are averages of 2–4 determinations at indicated times in 2–8 experiments. SD did not exceed ± 22% of each mean value.

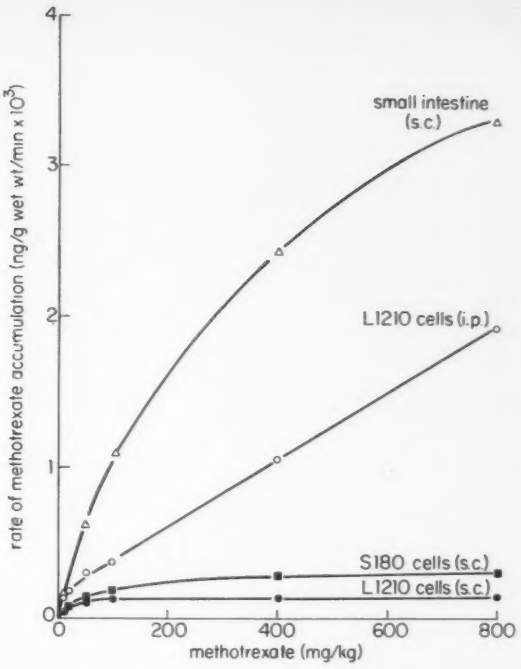

FIGURE 3.—Initial rate of MTX accumulation in L1210 and S180 cells and mouse small intestine after administration at varying doses. Values are averages of 2–4 determinations obtained in 2–3 experiments. SD did not exceed ± 25% of each mean value.

pendence to at least 400 mg/kg and maximum accumulation in gut was > 20-fold higher than in L1210 cells. Exchangeable drug is determined from a comparison of total drug accumulation to the dihydrofolate reductase binding content. At 3 and 12 mg/kg of MTX, loss of drug in gut occurs only to an equivalent of the enzyme content. At these doses in L1210 cells and at higher doses in both tissues, the level eventually assumed is always higher than this equivalent. As before (13), this may be accounted for by the binding of drug to newly synthesized enzyme. After 3 mg/kg, exchangeable drug is retained > 4 times longer in the L1210 cells than in the small intestine. The significance of this difference on a therapeutically optimum dosage schedule to the selective antitumor effects of MTX has already been discussed (12,13). As dosage is increased, the retention time increases in both tissues; however, the difference in retention in tumor versus gut diminishes considerably. At 96 mg/kg the difference is less than twofold.

Dose dependence for initial rates of uptake of drug in tumor and small intestine (fig 3) was evaluated by measurements made during the initial linear tissue course (< 40 minutes). Following dosages of 3–800 mg/kg given sc, saturation kinetics are seen for uptake in small intestine and tumor tissue (data for an S180 ascites sarcoma are also given). Also, there is clearly a difference in the degree of saturability for uptake by gut versus tumor cells. This difference probably reflects only the individual carrier-mediated transport capacities, since accumulation in plasma is still proportional to dosage in this range (14). The same relative saturability was shown in vitro (15,16) and uptake in vivo is inhibited by simultaneous CF (17). By giving drug ip a deviation from saturation kinetics for the rate of initial uptake could actually be demonstrated (*see* fig 3) at doses > 12 mg/kg with L1210 cells in the peritoneal cavity. This is indicative of the extracellular drug levels which must be achieved and sustained in order for passive diffusion to be important in respect to net permeation of drug.

Effects of MTX on DNA Synthesis

After a dose of ≥ 3 mg/kg, incorporation of labeled ^{3}H-UdR was undetectable (< 0.5% of control values) within 1–2 hours, and the same time course for inhibition was observed (also reported earlier, *see* refs 12 and 13); the duration of inhibition (fig 4)

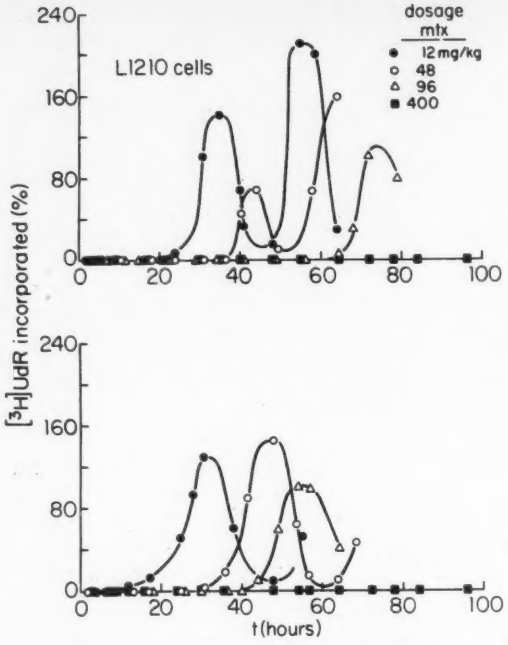

FIGURE 4.—Time course for recovery of DNA synthesis in L1210 cells and mouse small intestine after sc administration of MTX at varying doses. Values are averages based on 2–4 determinations for rate of ^{3}H-UdR incorporation into DNA in treated vs control animals obtained in 2 or 5 separate experiments. SD did not exceed ± 28% of mean values for absolute rate of incorporation.

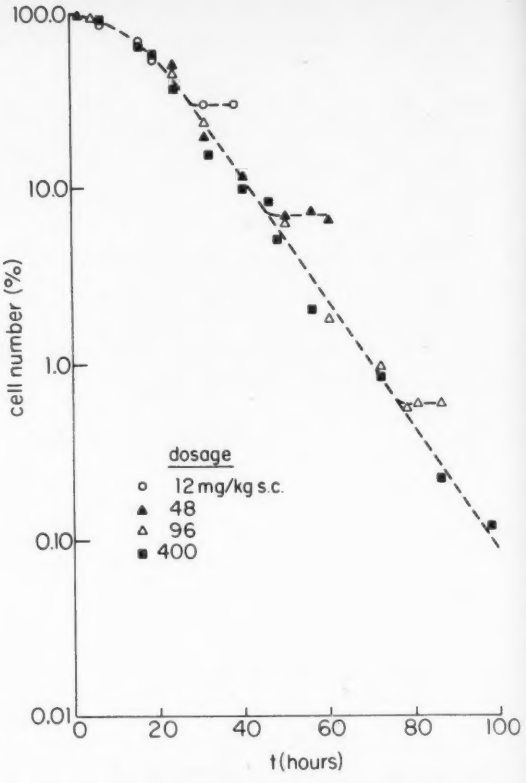

FIGURE 5.—Rate and extent of L1210 cell loss in the peritoneal cavity of leukemic mice receiving varying sc doses of MTX. Values are averages of 2–4 determinations at indicated times in 2–8 experiments. SD did not exceed ± 22% of mean values.

correlated closely with the extent of retention (figs 1 and 2) of exchangeable drug. Recovery in both L1210 cells and small intestine was highly synchronous. Overall, the onset of recovery in L1210 cells after each dosage (12, 48, and 96 mg/kg) agreed with the data on cell loss (fig 5) and occurred later than recovery in the small intestine. No recovery was observed in either tissue for a period of at least 96 hours when 400 mg/kg was given. When compared to the toxicity data given in a later section, it would appear that lethality from MTX is associated with a duration of inhibition of DNA synthesis in these mice of > 25–30 hours.

Pharmacokinetic and Biochemical Effects of CF

Following equivalent dosages of CF and MTX given simultaneously, there is a reduction (17) in the level of accumulation and persistence of drug in tumor and small intestine when compared to MTX alone, and a correspondingly shorter duration of inhibition of DNA synthesis. Since prior studies in mice (1–4) indicated that high MTX dosage regimens with delayed administration of CF (12–24 hours following MTX) prevented most toxicity and were more therapeutically effective than regimens incorporating simultaneous CF administration, experiments were initiated in which CF was administered 2 and 16 hours after MTX. At doses of ≤ 48 mg/kg, CF given sc 2 hours after 400 mg/kg of MTX had no effect on the retention of exchangeable drug in either L1210 cells or small intestine (fig 6). At doses of ≥ 96 mg/kg of CF, the rate of drug loss was more rapid and exchangeable drug levels persisted for much shorter periods of time. The effect of CF (24, 48, and 400 mg/kg sc) given 2 hours after 400 mg/kg of MTX on the inhibition and recovery of ^{3}H-UdR incorporation is shown in figure 7. Shortly after these doses of CF, a barely detectable effect is

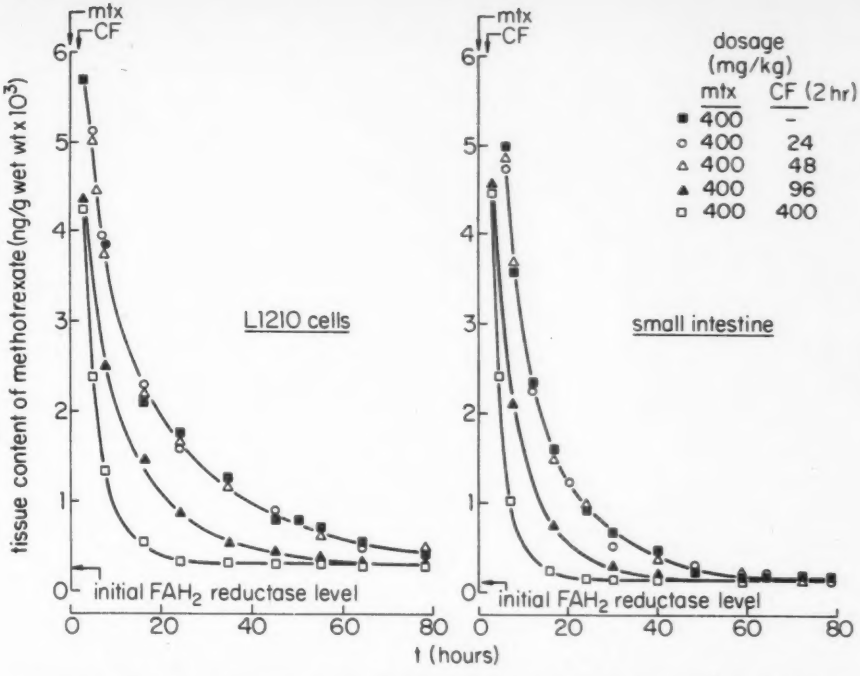

FIGURE 6.—Effect of varying doses of CF given 2 hrs after MTX on the retention of drug in L1210 cells and mouse small intestine. CF and MTX were given sc. Values are averages of 2–4 determinations made during 2–3 experiments. SD did not exceed ± 18% of the mean.

observed in both tissues. A cycle of incorporation is initiated within 30 minutes, which reaches a maximum of 3%–5% of control levels of incorporation by 4–6 hours, and then becomes undetectable again at 10 hours. Between 20 and 25 hours and only after 400 mg/kg of CF, a major cycle of ^{3}H-UdR incorporation is initiated in both L1210 cells and small intestine. This major cycle of synthesis is initiated when total drug levels in tissue approach a nonexchangeable level (fig 6). Toxicity is prevented only in those animals receiving 400 mg/kg of CF. The extent of tumor cell loss after 24 and 48 mg/kg of CF is the same as that seen with 400 mg/kg of MTX alone (fig 5). After 400 mg/kg of CF, tumor cell loss did not reach one log.

The administration of 400 mg/kg of CF sc 16 hours after 400 mg/kg of MTX (fig 8) resulted in a rapid loss of exchangeable drug in L1210 cells and small intestine. In both tissues, drug levels approached the dihydrofolate reductase content in 30 hours. The same effect of CF was obtained in both tissues at doses as low as 24 mg/kg. At 12 mg/kg, sc CF drug loss was not as rapid. The effect of CF in the same experiment on the inhibition of DNA synthesis by MTX is seen in figure 9. The incorporation of ^{3}H-UdR remained undetectable beginning after the first 90 minutes following 400 mg/kg of MTX given alone. Following the administration of 400 mg/kg of CF, a brief cycle of synthesis was detectable within 1 hour in L1210 cells and small intestine. Levels of incorporation reached a maximum of 3%–5% (4–6 hours) of control levels and by 6–8 hours, incorporation was again undetectable. At 19 hours after this same dose of CF, a major cycle of synthesis was initiated in both L1210 cells and small intestine, which reached a peak at about 40 hours after CF. After 24 mg/kg of CF, a brief cycle of synthesis was also initiated within 1 hour, but only in L1210 cells. In the small intestine, a major onset of recovery of synthesis was observed within 24 hours. This occurred as a minor cycle of synthesis to a maximum of 25% of control levels of incorporation within 7 hours followed by a major cycle of synthesis beginning 40 hours after CF. Maximum levels of incorporation reached in the second cycle were comparable to levels achieved with 400 mg/kg of CF. In L1210 cells, after 24 mg/kg of CF, only a minor cycle of synthesis was observed after 24 hours. Incorpora-

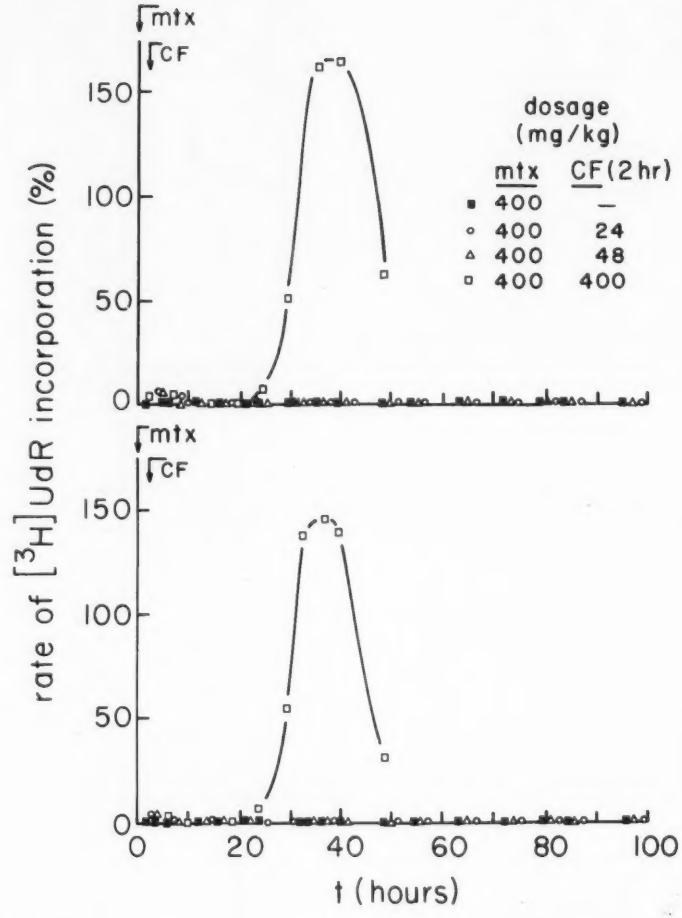

FIGURE 7.—Effect of CF given 2 hrs after varying doses of MTX on the inhibition of DNA synthesis in L1210 cells and mouse small intestine. CF and MTX were given sc. Values are based on 2–4 determinations for rate of ^{3}H-UdR incorporation into DNA of drug treated vs control animals obtained in 3 experiments. SD did not exceed ± 20% of each mean for absolute rates.

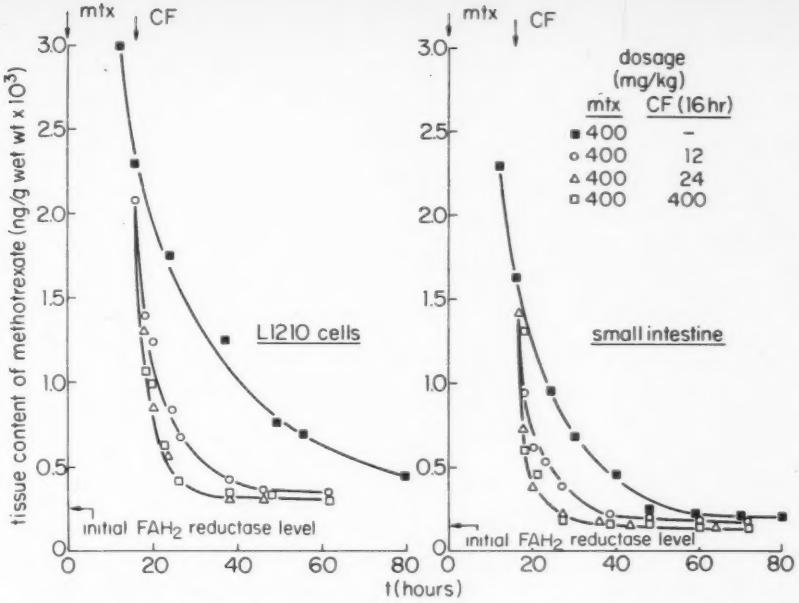

FIGURE 8.—Effect of varying doses of CF given 16 hrs after MTX on the retention of drug in L1210 cells and mouse small intestine. CF and MTX were given sc. Values are averages of 2–4 determinations in 5 experiments. SD did not exceed ± 25% of each mean value.

tion never exceeded 10% of control levels and was undetectable within 12 hours and remained so for at least 65–75 hours.

A major resumption in DNA synthesis in L1210 cells and small intestine after 400 mg/kg of CF and only in small intestine after 24 mg/kg of CF is coincident with the approach of drug levels to the equivalent dihydrofolate reductase content in these tissues. The differences in the extent of recovery of DNA synthesis in L1210 cells following 24 and 400 mg/kg of CF given 16 hours after MTX are reflected in the data on tumor cell loss given in figure 10. With drug alone at 400 mg/kg sc, at least a three-log decrease in cell number in the peritoneal cavity can be demonstrated during a period of 100 hours. Following 400 mg/kg of sc CF 16 hours after drug, the total cell loss did not reach one log. However, after 24 mg/kg of sc CF, total loss was two logs in magnitude.

Therapeutic Efficacy of High-Dose MTX With Delayed Low-Dose CF

A preliminary evaluation of a regimen of therapy employing low doses of CF (6–48 mg/kg sc) given 16 hours after a single dose of 400 mg/kg of MTX sc in both L1210 and S180 murine tumor models is shown in table 1. This dose of MTX alone is lethal. Lethal toxicity was avoided in approximately 80% of animals receiving MTX and a single dose of 24 mg/kg of CF. The antitumor effects on this schedule were twofold (L1210) or threefold (S180) greater than the effects obtained at the approximate equitoxic dosage of MTX alone (48 mg/kg). More toxicity was observed at CF doses < 24 mg/kg and no further decrease in toxicity was associated with higher CF dosages. The antitumor effects were the same at either 24 or 48 mg/kg of CF, but were diminished twofold at 400 mg/kg of CF. Toxicity was avoided entirely with no diminution of antitumor effects by a regimen incorporating three doses of CF at 24 mg/kg (16, 20, and 24 hours after drug) or two doses at 48 mg/kg (16 and 20 hours after drug). Although the antitumor effects obtained were unchanged with respect to regimens incorporating one dose of CF, the effect obtained relative to that seen at the maximum tolerated dosage of MTX alone (24 mg/kg) was now threefold (L1210) and sixfold (S180) lower.

DISCUSSION

A correlation is defined over a wide dosage range between the persistence of exchangeable MTX and the extent and duration of inhibition of DNA syn-

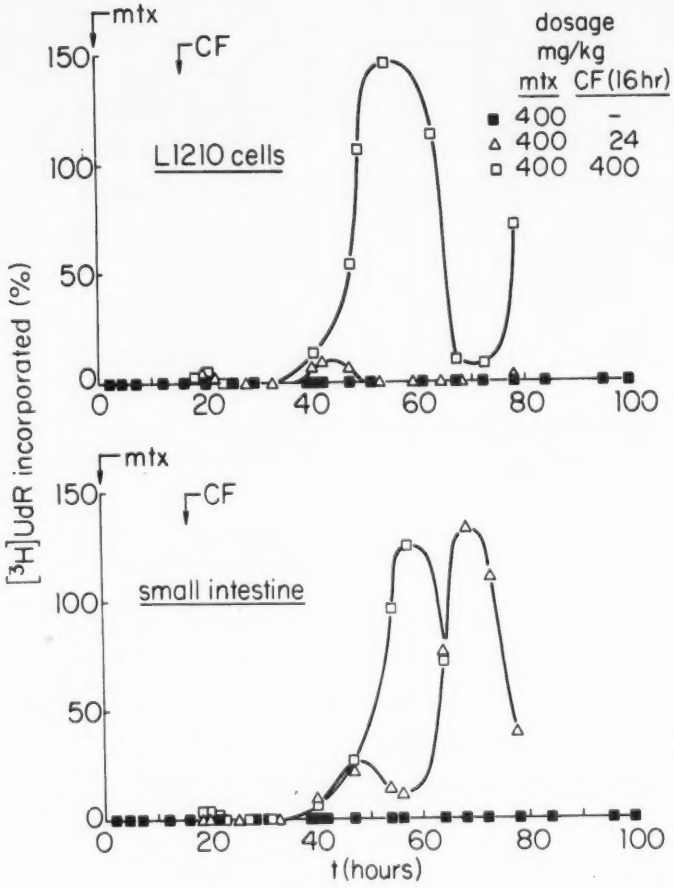

FIGURE 9.—Effect of varying doses of CF given 16 hrs after MTX on the inhibition of DNA synthesis in L1210 cells and mouse small intestine. CF and MTX were given sc. Values are averages of 2–4 determinations made in 4 separate experiments. SD did not exceed ± 28% of each value for absolute rate of incorporation.

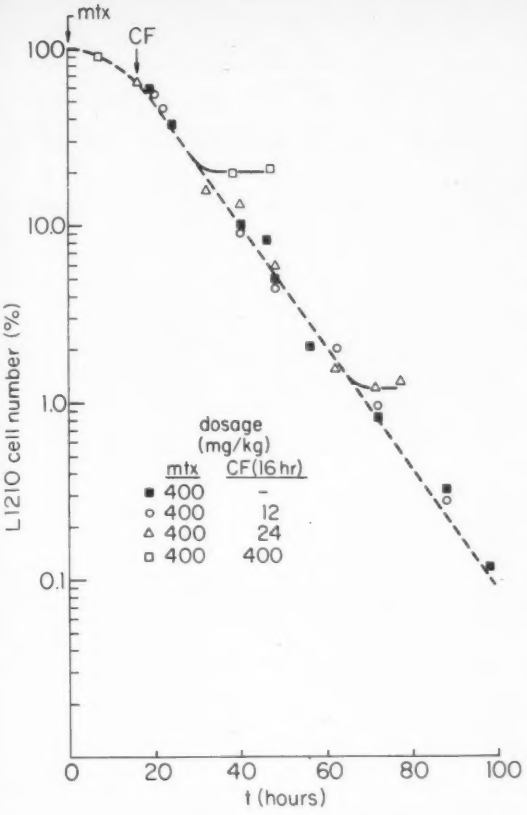

FIGURE 10.—Effect of varying doses of CF given 16 hrs after MTX on the extent of L1210 cell loss from the peritoneal cavity of leukemic mice. CF and MTX were given sc. Values are an average based on 2–3 determinations in 4 experiments. SD did not exceed ± 23% of mean value.

thesis in both tumor tissue and small intestine, the drug-limiting organ in mice. The dose dependence for the extent of tumor cell kill observed here apparently has the same biochemical basis reported previously (9) for the extent of drug-related pathology in small intestine, ie, the duration of inhibition of DNA synthesis.

The saturation kinetics for initial uptake of MTX into tissue is of significance with respect to high-dose MTX therapy with CF. The vast majority of drug accumulation within the dosage range studied must occur within the constraints of the individual carrier-mediated mechanisms, since plasma levels of drug, required to allow for appreciable entry of drug by passive diffusion, are not achieved. The changing relationship in tumor versus small intestine with respect to the retention time for drug and duration of inhibition of DNA synthesis as dosage is increased also suggests that the difference observed at the optimum therapeutic dosage range, which accounts (13) for the responsiveness of some tumors, is probably irrelevant to therapy at higher dosages.

The effect of CF on the retention of drug in tissue can be attributed to competition between both for a carrier entity necessary for the continuing influx of drug and the difference in the dose dependence for this effect at 2 and 16 hours accounted for by a drastic difference in plasma levels of MTX at these times. CF-mediated recovery of DNA synthesis in L1210 cells and small intestine occurs under two different circumstances. As with that seen with drug alone, recovery occurs in a synchronous fashion. An initial cycle of synthesis of extremely low magnitude occurred almost immediately and is unrelated to any obvious effect on tissue levels of drug. It is doubtful that this cycle of synthesis has any pharmacologic significance.

The major biochemical effect of CF appears to be a direct consequence of the pharmacokinetic effect achieved in tissue. When a more rapid rate of loss of drug from tissue was induced, an earlier onset of recovery of DNA synthesis at a substantial level was the result. In this respect, effects of CF given at 2 or 16 hours after drug appear to be the same in L1210 cells and small intestine with one major exception. Both 24 and 400 mg/kg of CF at 16 hours after MTX achieve an identical pharmacokinetic effect in these tissues. However, a major recovery in DNA synthesis in L1210 cells occurred only after 400 mg/kg of CF. These results agree not only with the data on tumor cell kill but the data derived from a single course of therapy as well.

It would appear that some difference exists in L1210 cells (and probably S180 cells) versus small intestine which determines the ability for resuming folate coenzyme synthesis after a prolonged duration of inhibition. Thus, this explains the necessity for high doses of CF for recovery in L1210 cells, even though CF at 24 and 400 mg/kg mediates an identical rate of drug loss from tissue. These findings will probably require confirmation at a biochemical level before their exact significance to CF rescue in general can be determined.

TABLE 1.—Relative therapeutic efficacy of high-dose MTX alone and with CF 16 hrs later

		L1210 leukemia‡				S180‡			
		Toxic deaths		Survivors§		Toxic deaths		Survivors§	
MTX* (mg/kg)	CF† (mg/kg)	No.	%	MST (days)	ILS (%)	No.	%	MST (days)	ILS (%)
—	—	0/45	—	6.8±0.5	—	0/45	—	11.2±1.4	—
24	—	1/15	6.7	9.4±1.0	39.8	0/15	0	12.9±1.5	15.2
48	—	3/15	20.0	10.1±0.7	44.5	4/15	26.7	14.4±1.9	35.6
96	—	7/15	46.7	12.9±1.2	88.3	8/15	53.2	19.7±2.3	67.2
125	—	10/15	66.7	12.0±0.9	76.2	10/15	66.7	21.1±1.9	88.0
250	—	15/15	100	—	—	15/15	100.0	—	—
400	—	35/35	100	—	—	35/35	100.0	—	—
400	6	14/15	93.5	—	—	13/15	86.7	22.7±3.0	102.6
400	12	11/15	73.5	12.7±1.0	86.5	10/15	66.7	23.6±2.6	110.1
400	24	10/45	22.2	12.9±1.3	88.3	9/45	20.0	22.8±1.8	103.0
400	48	5/30	16.6	13.0±0.7	91.3	6/30	20.0	22.5±2.1	101.3
400	400	3/20	15.0	9.8±0.9	44.2	3/20	15.0	17.4±2.8	58.2
400	24×3	0/20	0	12.8±0.8	86.8	0/20	0	23.9±2.3	113.0
400	48×2	0/20	0	12.3±0.7	81.0	0/20	0	22.3±2.6	98.4

*1 dose sc.
†1 dose sc 16, or 16 and 20, or 16, 20, and 24 hrs after MTX.
‡Average of 3–9 experiments.
§Animals not dying of toxicity; MST = mean survival time ± SD, and ILS = increase in lifespan.

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Ligand-Binding Radioassay for the Antifolate Compounds: Application in Patients Receiving Methotrexate [1,2]

Sheldon P. Rothenberg,* Maria da Costa, and M. Perwaiz Iqbal [3]

SUMMARY

The general principles of ligand-binding radioassays are reviewed and a specific noncompetitive system is described for the measurement of methotrexate (MTX) using dihydrofolate reductase as the binding determinant. This type of radioassay can also be exploited to measure any antifolate compound which binds to this enzyme even if [^{3}H]MTX is used as the tracer. The radioassay has now been used to measure MTX in all types of body fluids, tissue extracts, and rbc lysates from patients receiving this drug.

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The pharmacokinetics of methotrexate (MTX) in man has been the subject of a number of studies to establish the absorption, plasma clearance, and patterns of excretion (1–5). Yet, there has been a lack of data correlating the concentration of these drugs in biologic fluids and tissues with their therapeutic and toxic effects. This has probably been due to the inadequate sensitivity and technical difficulty of assay procedures which made systematic and routine measurements of drug concentration difficult in patients receiving these chemotherapeutic agents.

Because of the observation that neurotoxicity of MTX can be correlated with the rate of clearance of the drug from the cerebrospinal fluid (CSF) (6) and experimental observations indicating that the pharmacologic effect of this drug can be correlated with the blood concentration (7), there has been a renewed interest in measuring MTX in body fluids. An additional impetus for a practical assay procedure to measure the antifolate drugs has been the recent use of high-dose MTX for the treatment of certain malignancies, because the rate of clearance from the blood may be valuable as a guide in predicting and managing toxicity.

A number of methods which include microbiologic (8), spectrofluorometric (9–11), and enzyme inhibition assays using spectrophotometric (12) and radioassay (13) procedures have been used to measure MTX. Ligand-binding radioassay methods which are more sensitive and technically easier have recently been developed because of the availability of high specific activity [^{3}H]MTX. The purpose of this paper is to explain the principle of these radioassay procedures and to summarize some of our early results using one specific method to measure MTX in body fluids and tissues.

General Principles of a Ligand-Binding Radioassay

Ligand-binding radioassay methods are based on the principle of radioimmunoassay (14), where an isotopically labeled antigen and an unlabeled antigen compete for binding sites on a specific antibody to the antigen. The more general term "ligand-binding radioassay" has been used where the macromolecular binder is a protein other than an antibody. The principle of ligand-binding radioassay may be best understood from the following equations:

$$[L^*] + [B] \rightleftharpoons [L^*\text{–}B]$$

$$+$$

$$[L]$$

$$\uparrow\downarrow$$

$$[L\text{–}B]$$

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[1] Supported by Public Health Service grant CA-08976 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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where L* is the isotopically labeled ligand, L is the unlabeled ligand in the standard or sample being assayed, and B is a macromolecular binder of the ligand.

It can be seen from these two equations that if the concentration of B is sufficient to bind only a fraction of L*, then the addition of L to the reaction mixture will compete for binding sites on B and less of L*–B will form. The inverse relationship of L to L*–B can be expressed graphically as a dose-response curve which is then used as a reference standard to determine the concentration of L in unknown solutions which are assayed in the exact system as the standards.

The technical requirements for these radioassay procedures are: (*a*) an isotopically labeled ligand with a specific activity high enough to use a tracer concentration in the reaction mixture which approximates the lowest concentration of the ligand to be assayed; (*b*) a binder with a high affinity for the ligand (it has been established that the higher the equilibrium constant for the reaction, the greater the sensitivity of the radioassay [14]); (*c*) a precise method of separating the bound and free ligand so that the reaction can be quantified; and (*d*) standardization of the conditions which may affect the interaction of the ligand with the binder such as pH, buffer ions, cofactors, and protein concentration, and to ensure the exact same conditions for assaying the unknown as for obtaining the standard reference curve.

The term "ligand" is a descriptive one used to describe a small molecule which binds to a larger macromolecule. If the macromolecular binder is an antibody, then the more specific term "radioimmunoassay" is used to describe the radioassay. If the binder is another type of binding macromolecule (ie, physiologic binding proteins such as transport, receptor, or enzyme), then the more general term "competitive ligand-" or "protein-binding radioassay" is generally used.

"Competitive" and "noncompetitive radioassay" are other terms frequently used to describe radioassay procedures. A competitive ligand-binding radioassay is one in which the binder reagent is added to a mixture of the isotopically labeled ligand and the unlabeled ligand. A noncompetitive ligand-binding radioassay is a two-phase system in which the binder is first incubated with the standard unlabeled ligand or the sample containing an unknown concentration of the ligand, followed in the second phase by the isotopically labeled ligand. The amount of labeled ligand which couples to the binder is, in effect, a measure of residual unoccupied sites on the binding macromolecule. The chief disadvantage of the noncompetitive system is that it requires two technical manipulations. However, this assay system is significantly more sensitive than the competitive type of radioassay.

Ligand-Binding Radioassay to Measure MTX

For the application of the ligand-binding procedure to measure MTX, [^{3}H]MTX is the labeled ligand and the dose-response standard curve is obtained using unlabeled MTX as the competing unlabeled ligand. The macromolecular binder may be dihydrofolate reductase (DHFR) from any source since this enzyme has a high affinity for this antifolate compound. Alternatively, the binding macromolecule may be an antibody obtained by immunizing animals with MTX, as a hapten covalently coupled to an immunogenic macromolecule (15–17). The variable properties of the antibody population obtained, and the uniform properties and availability of DHFR from a variety of tissues such as L1210 leukemia cells, liver, or bacteria, would favor the use of the enzyme as the binder reagent for the radioassay.

To ensure the maximal binding activity and affinity of DHFR for MTX, the reaction mixture should contain at least 5×10^{-5} M NADPH and 5×10^{-3} M 2-mercaptoethanol. Whereas the 2-mercaptoethanol appears to stabilize the binding capacity of the enzyme, the NADPH has an effect on both the binding capacity of DHFR and the equilibrium constant for the binding of MTX.

The optimal pH to obtain the highest binding affinity for the reaction between DHFR and MTX should be between 4.8 and 6.0. Although for our initial studies we used a pH of 6.0, subsequent experiments have indicated that the binding is more precisely stoichiometric at pH 4.8.

The amount of charcoal is also critical since an excess can adsorb some DHFR which is bound to MTX. Neutral Norit A charcoal suspended in 0.5% dextran-10 (molecular weight averaging 10,000) will adsorb significant amounts of bound MTX if the final concentration is > 4 mg/ml of reaction mixture.

Assay Method Using DHFR

The details of this method using DHFR from L1210 leukemia cells have been reported previously (18). If this leukemia strain is being maintained in the laboratory, it is simple to collect a large volume of cells and prepare a pooled lysate. These lysates can be stored in a frozen state for prolonged periods without loss of activity.

Alternatively, any source of DHFR can be used. Particulate-free supernates of crude homogenates from avian or mammalian liver could be used be-

cause these tissues contain a high concentration of DHFR. A bacterial source of this enzyme has also proven suitable (19).

To obtain maximum sensitivity, several principles should be followed: (*a*) use the lowest amount of [^{3}H]MTX which will give an adequate counting rate with the liquid scintillation detector available; (*b*) dilute the DHFR in 5% bovine albumin and use an amount which binds 50%–60% of the tracer [^{3}H]MTX; (*c*) use a buffer of pH 4.8–5.0 containing the required concentrations of NADPH and 2-mercaptoethanol; (*d*) use a sequential noncompetitive reaction system where the unlabeled MTX standards or the unknown sample is first incubated with the diluted binder reagent followed by the incubation with the [^{3}H]MTX; and (*e*) separate the bound from free MTX using ≤ 4 mg of charcoal from a suspension in dextran-10.

Results and Discussion

Figure 1 compares dose-response curves using a competitive and noncompetitive sequential system. The difference in sensitivity between the two curves is striking. The high sensitivity of the noncompetitive system permits the assay of small volumes of serum. This is important because the binding of MTX by DHFR is influenced by the protein concentration of the reaction mixture. This is shown in figure 2. The addition of 0.1 ml of serum increased the apparent binding of [^{3}H]MTX by the L1210 lysate for the same incubation time period. This was not observed with 0.05 ml of serum. This was not due to incomplete extraction of free [^{3}H]MTX nor to the additional binding of MTX by serum proteins. Obviously, if this effect is variable with different serums, it will introduce an error into the assay even if the standard curve is assayed with normal serum from a single source. Therefore, if the expected concentration of MTX in the unknown serum is low enough to require the direct assay of > 0.05 ml, the serum should be extracted as previously described (18).

Tissue homogenates, cell lysates, rbc hemolysates, or other material containing high protein or bound MTX should be similarly extracted by boiling with the assay buffer to release the MTX and precipitate most of the proteins.

The dose-response curve can be linearized by plotting the reciprocal of the fraction bound against the MTX standards as shown in figure 3. In general, the curve remains linear up to values of 160 pg of unlabeled MTX. For values above this, it is necessary to assay higher dilutions of the sample. The advantage of a linear reference curve is that a few experimental points can be used to calculate the regression equation by a least-squares analysis using a desk-top computer.

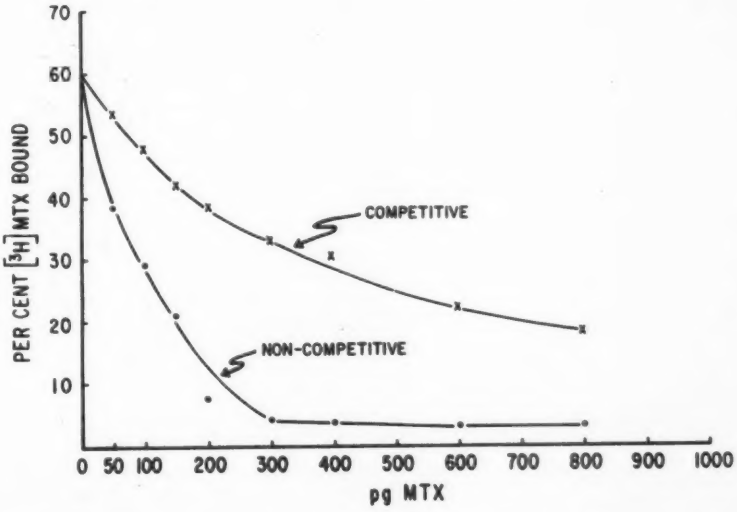

FIGURE 1.—Comparison of dose-response standard reference curves using a competitive and a sequential noncompetitive assay system. With a competitive system the binder reagent is added to a mixture of [^{3}H]MTX and the unlabeled MTX standards. With a noncompetitive system, the binder reagent is first incubated with the unlabeled MTX (or the sample being assayed) followed by the incubation with [^{3}H]MTX.

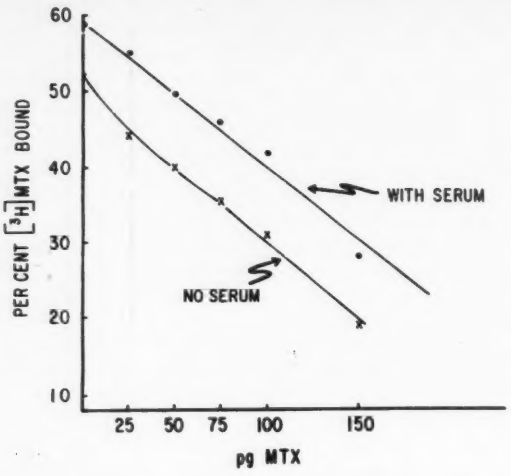

FIGURE 2.—Effect of serum proteins on the dose-response curve. The reactions were identical except that 0.1 ml of serum was added to 1 set of reaction tubes. This effect was not observed with 0.05 ml of serum.

It should be appreciated that the standard antifolate compound need not be MTX even though [^{3}H]MTX is used as the tracer. Any antifolate compound may be assayed against [^{3}H]MTX to obtain the dose-response curve which is then used to determine the concentration of that antifolate compound in biologic fluids or tissues. If the antifolate compound has a lower affinity for DHFR than [^{3}H]MTX, then the sensitivity of the reference curve may be less than that obtained with unlabeled MTX. Nevertheless, the sensitivity may still be adequate for a given clinical or experimental purpose, particularly if a noncompetitive sequential procedure is used. An example of this application is shown in figure 4 where pyrimethamine, a weaker folate antagonist than MTX, was assayed against [^{3}H]MTX as the tracer. A dose-response curve was obtained ranging from 25 to 300 ng of the drug. Although this is about 1000-fold less sensitive than that obtained with unlabeled MTX, it may be sufficient to measure the concentration of pyrimethamine for clinical purposes.

The application of the radioassay to measure MTX in the serum of a patient given 3.5 g by a 6-hour iv infusion followed by citrovorum factor res-

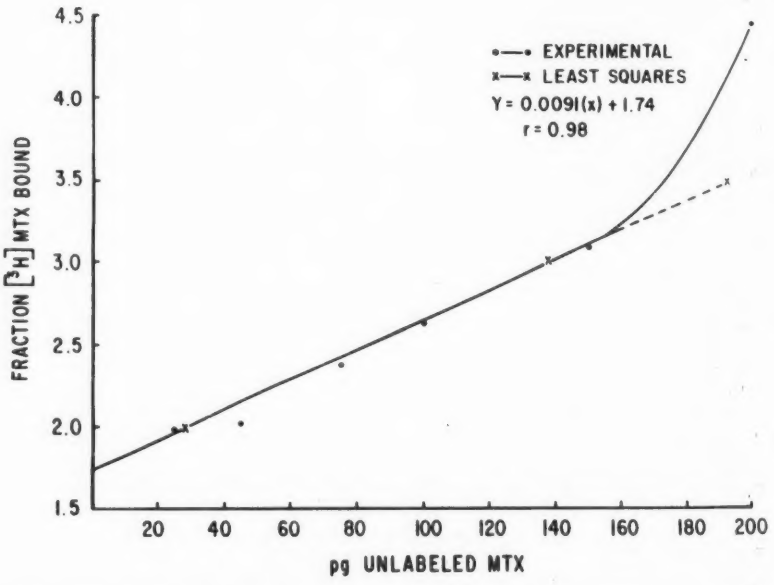

FIGURE 3.—Typical dose-response curve. If the sample aliquot to be assayed is expected to contain > 160 pg of MTX, it is best to dilute it so that the assayed value falls on the linear part of the curve.

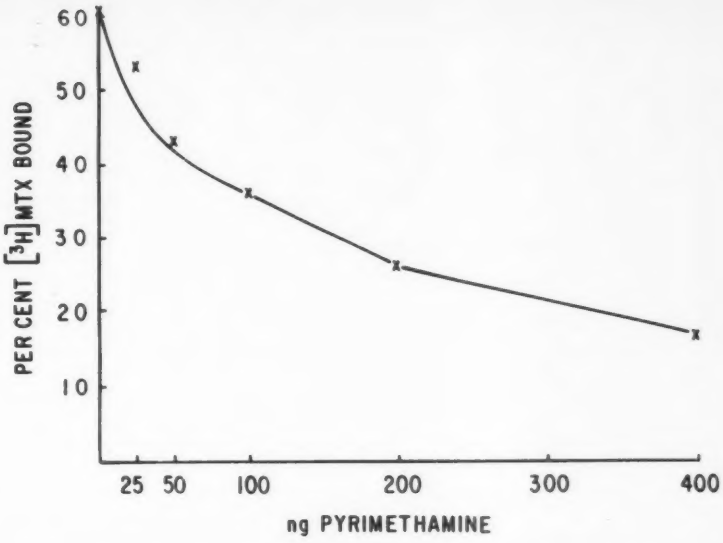

FIGURE 4.—Dose-response curve obtained using pyrimethamine instead of unlabeled MTX as the reference standard. The reactions contained the same [^{3}H]MTX as used for the unlabeled MTX reference curves.

cue is shown in figure 5.[4] This patient had endocervical carcinoma with ascites and hepatic metastasis and, subsequent to the MTX therapy, developed renal shutdown. Although hemodialysis was started, the large reservoir of MTX in the ascitic fluid prevented a continuous reduction in the serum MTX concentration which appeared to remain constant at about 10^{-4} M after the initial decrease.

Tumor tissue and kidney homogenates from this patient were subsequently assayed for MTX. The extracts were assayed before and after 24 hours of dialysis against buffer to determine the total and protein-bound drug (presumably to DHFR). The results are shown in figure 6. The total drug concentration per mg of protein in the tumor was 360 ng/mg of protein before dialysis and 2.6 ng/mg of protein following dialysis. In the kidney, the total MTX was 11,000 ng/mg of protein before dialysis and 41 ng/mg of protein following dialysis. The percent of protein-bound drug was 0.26 in the tumor and 0.37 in the kidney. The higher concentration of MTX in the kidney was not due to greater contamination with blood since there was actually 50% more benzidine-reacting material in the tumor extract. Rather, the higher free-MTX content in the kidney represented drug precipitated in the tubules and probably in the interstitial space.

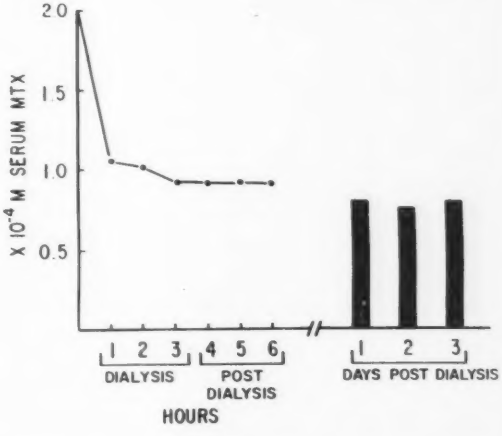

FIGURE 5.—Clearance of MTX from the blood of a patient given 3.5 g for the treatment of metastatic endocervical carcinoma with ascites. The patient was also treated by hemodialysis when she developed renal shutdown.

In order to determine whether there is any correlation between the serum MTX concentration and the multiple-dose administration of this drug, we have been routinely assaying blood from patients

[4]The serum and tissue samples from this patient were obtained through the courtesy of Dr. Samuel Waxman, Mt. Sinai School of Medicine, New York, NY.

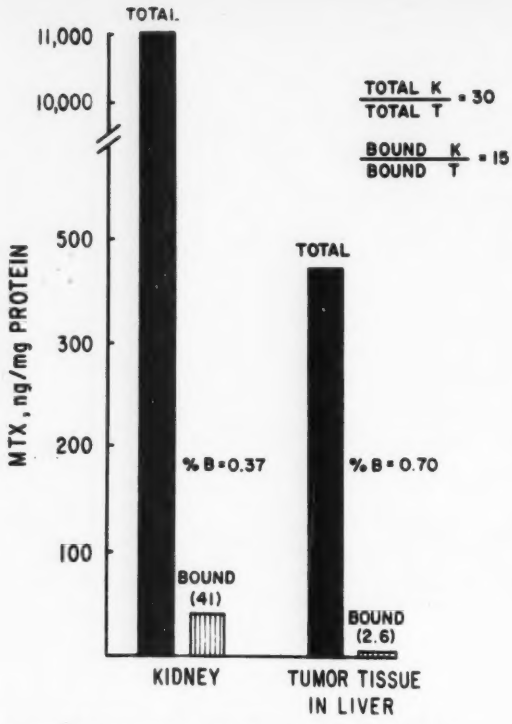

FIGURE 6.—Assay of free and bound MTX in kidney and tumor tissue of the patient treated with high-dose MTX.

receiving MTX according to a variety of therapeutic protocols. The results of one representative study in women with breast cancer receiving 25 mg of MTX iv weekly are shown in figure 7. Samples of blood were obtained 7 days after the previous dose and just prior to the next dose. No pattern of serum drug concentration could be observed in these representative patients shown, as well as in several other patients similarly treated. At times, the MTX concentration appeared to be zero (or < 250 pg/ml which is the absolute sensitivity of the radioassay) and at other times, the concentration appeared to be easily measurable ranging from 300 to 3500 pg/ml. This variation in serum MTX concentration could be due to the periodic release of the drug from various tissue stores.

In contrast to these results, a patient who received 5–25 mg of oral MTX twice weekly as part of a maintenance protocol for acute myelogenous leukemia had more consistently measurable serum MTX in samples taken 48 hours after the last dose (fig 8). During a period of oral toxicity, he had relatively high levels of serum MTX of 138 and 3.6 ng/ml.

On four occasions, we also measured the 48-hour cumulative urinary excretion of MTX in this patient, and the results are shown in figure 9. As previously observed by Henderson et al (4) with their studies in man, about 50% of the administered dose of MTX was excreted over each subsequent 48-hour collection period, indicating that the absolute amount of MTX retained at each dose level in-

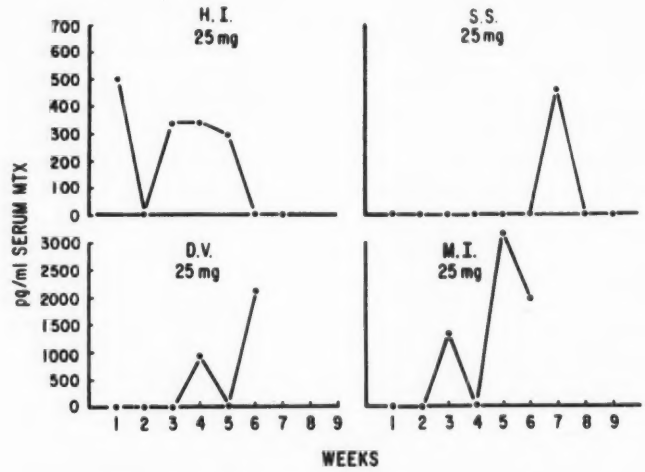

FIGURE 7.—Serum MTX concentration in women with breast cancer 1 wk after receiving 25 mg iv. A sample of blood was obtained just prior to the administration of the next dose.

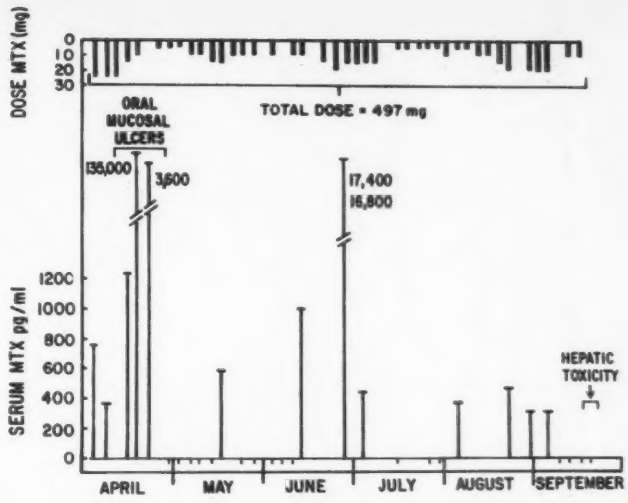

FIGURE 8.—Correlation of oral MTX with serum concentration. A sample of blood was taken 48 hrs following the last oral dose. At the time clinical hepatic toxicity was observed, no MTX could be measured in the serum.

creased. If the stool excretion remained constant at about 10% for these dosage levels (4), then it is possible to roughly calculate that the total MTX retained was 175 mg out of a total dose of 497 mg given during the 6-month period of treatment.[5] The MTX was discontinued when this patient developed a marked disturbance of hepatic enzymes and a liver biopsy showed toxic hepatitis.

At the time of the liver biopsy, a sample of tissue was also extracted and assayed for MTX; the results are shown in figure 10. The total MTX in the liver extract, at the time the serum contained no measurable amount of drug, was 4.3 ng/mg of homogenate protein. Based on an average liver weight of 1500 g, the total hepatic MTX could be estimated to be about 6.0 mg which represents approximately 3.5% of the retained MTX.

To investigate further the nature of the hepatic MTX, a sample of the biopsy extract was dialyzed for 24 hours against phosphate buffer, pH 6.0. It can be seen from figure 10 that most of the MTX was dialyzable. This might explain the observed hepatic toxicity since the pharmacologic (and, therefore, the toxic) effect of MTX may be enhanced by a high intracellular concentration of free drug which would ensure saturation of low-affinity binding sites on DHFR (20).

[5]An additional 5% of each dose was assumed to be excreted during the subsequent days following the 48-hr collection period (3).

Another tissue which accumulates MTX is the erythrocyte. This initial accumulation of MTX in rbcs appears to be by passive diffusion along a concentration gradient as shown in figure 11. Within 1 minute after administration of MTX iv, the drug can be assayed in washed rbcs and the concentration then decreases over the subsequent 18 hours as the plasma MTX concentration decreases. However, even when the MTX is not measurable in the plasma, a constant amount of MTX can be assayed in the rbcs. We have consistently found 1–3 ng of MTX/ml of rbcs 1 week after a single administration of MTX iv. Since there is only a small accumulation of DHFR in rbcs of patients treated with MTX (21), most of the MTX observed in these cells is probably free. Under such conditions the slow efflux of MTX from these cells might serve as a type of reservoir. The kinetics of in vivo and in vitro accumulation and release of MTX by erythrocytes is now under investigation.

The preceding studies using the ligand-binding radioassay to measure MTX in various body fluids and tissues required a dose-response reference curve to determine the concentration of the drug in unknown samples. Most recently, however, we have established the conditions whereby MTX may be assayed by the principle of stoichiometric isotope dilution. By this method, the amount of unlabeled ligand can be calculated from the quantity of the tracer ligand displaced from the reagent binder and a dose-response reference curve is unnecessary.

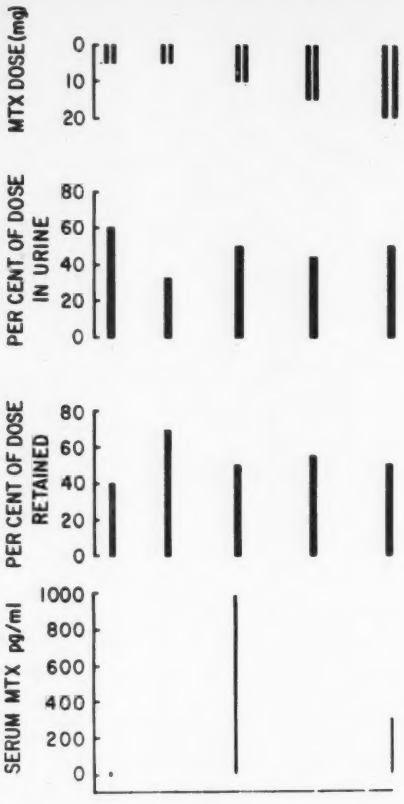

FIGURE 9.—Correlation of urinary excretion and dose of MTX in a patient receiving MTX twice weekly. Each collection was for 48 hrs starting on the day of administration. The % of the dose retained was calculated from the dose given and the urinary excretion. No correction was made here for the excretion of MTX in the stool. (*See* text.)

In order to measure MTX by stoichiometric dilution analysis, two conditions must be satisfied. First, the quantity of tracer used must be precisely known. Second, the binding capacity of the DHFR must remain constant at saturating concentrations of MTX. For this latter requirement to be satisfied, the reaction between the MTX and DHFR must be irreversible (or pseudo-irreversible) as described by Werkheiser (22). Under these conditions the fraction of total MTX bound is stoichiometrically proportional to the total MTX in the reaction mixture, and

$$B\,[T+U] = C \qquad (1)$$

where B is the fraction of total MTX bound, T is the quantity of [^{3}H]MTX, U is the quantity of unlabeled

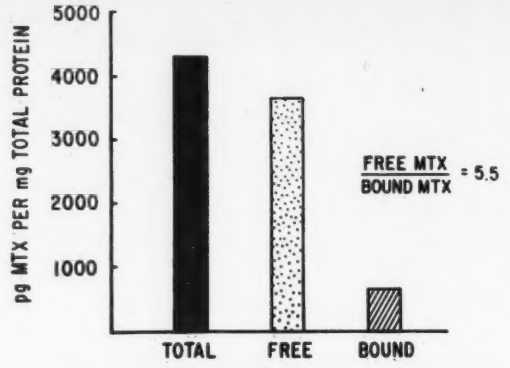

FIGURE 10.—Total free and bound MTX in the liver biopsy of the patient receiving oral MTX twice weekly as part of a maintenance protocol for acute myelogenous leukemia. (*See* text for method of determining each fraction.)

MTX (or the amount of MTX in the unknown sample), and C is the binding capacity of the DHFR used in the reaction; then

$$U = \frac{C - BT}{B}. \qquad (2)$$

These requirements can be satisfied by carrying out the reaction in 0.5 ml of 0.05 M citrate buffer, pH 4.8, and separating the free and bound MTX with 0.4 ml of a 1% Norit A charcoal suspended in 0.5% dextran-10. The previously indicated concentration of NADPH and 2-mercaptoethanol should be used and the reaction is the competitive type where the binding lysate is added to a mixture of [^{3}H]MTX and the sample to be assayed.

The data in table 1 show the results of this type of radioassay. The fraction of [^{3}H]MTX bound (B) (which also represents the fraction of total MTX bound) is determined experimentally. The binding capacity (C) is calculated from the fraction of [^{3}H]MTX bound in the absence of the sample being assayed. For this experiment, 335 pg of [^{3}H]MTX was used and 60% was bound indicating that the binding capacity was 200 pg. As unlabeled MTX was added to each reaction mixture, the fraction of [^{3}H]MTX bound was determined and used to calculate the unlabeled MTX by equation 2. These calculated values are compared to the actual amount of unlabeled MTX used in each reaction. The mean value for the calculated MTX as a percentage of the expected was 91%. Thus, this type of radioassay could greatly simplify the routine measurement of MTX in biologic samples because it is a simple competitive reaction system and eliminates the need for a standard dose-response reference curve.

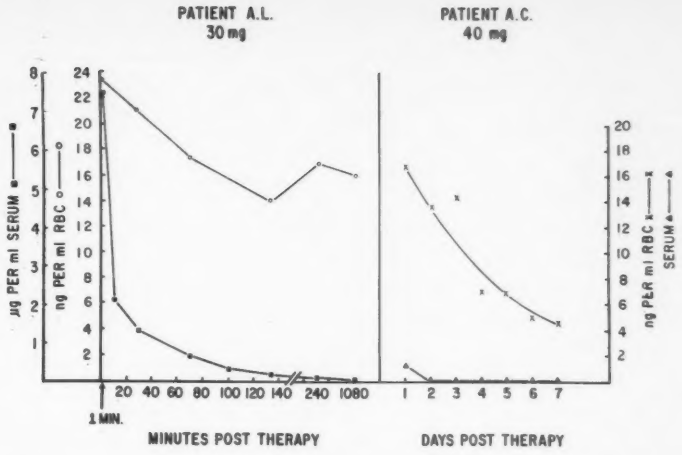

FIGURE 11.—MTX in washed erythrocytes and serum from 2 patients who received a pulse injection iv of MTX. The rbcs were washed 3 times with 0.9% saline before they were lysed with distilled water, extracted, and assayed.

TABLE 1.—Stoichiometric isotope dilution radioassay for MTX

MTX added (pg)	[^{3}H]MTX bound (%)	MTX calculated*	Calculated MTX/ added MTX
50	53	42	0.84
100	48	82	0.82
200	38	191	0.95
600	22	574	0.96
800	18	776	0.97
			Mean = 0.91

*Calculated by the formula:

$$U = \frac{C - BT}{B}.$$

U = unlabeled MTX; C = binding capacity of the diluted L1210 lysate (200 pg); T = amount of [^{3}H]MTX in the reaction (335 pg); and B = fraction of bound [^{3}H]MTX determined experimentally.

The development of these ligand-binding radioassays should facilitate the acquisition of more data concerning the clearance and distribution of MTX during the routine clinical use of this drug because this type of assay system is easily adapted to isotope laboratories already involved in numerous types of similar radiometric procedures. Although the value of such information may be debated on the basis of the pharmacokinetic information already known about MTX, the importance of knowing the CSF MTX concentration during repeated administration of the drug intrathecally, and of following the clearance of MTX from the blood of patients on high-dose protocols, appears to be of more certain benefit in predicting and managing toxic complications. Hopefully, clinical centers using these special therapeutic protocols will find this new method of assaying MTX of benefit.

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Radioimmunoassays for Methotrexate, Citrovorum Factor, Neocarzinostatin, and Actinomycin D [1,2]

Vic Raso [3,*]

SUMMARY

Antisera specific for methotrexate, citrovorum factor, actinomycin D, and neocarzinostatin have been elicited in rabbits by immunization with appropriate antigens. The antibodies have been used together with radiolabeled marker haptens to produce sensitive and specific radioimmunoassays for each of these drugs.

[Cancer Treat Rep 61:585–590, 1977]

Radioimmunoassay procedures are particularly suited to the task of monitoring drug levels in patients in a noninvasive manner. The applicability of such a technique is even more pronounced considering the large array of pharmacologic agents which are presently used for cancer chemotherapy. The specificity and flexibility of the immune response allow for the induction of antibodies which are tailor-made to selectively recognize almost any drug molecule. Radioimmunoassay techniques deploy such antibodies as the basis of a competitive binding assay and facilitate direct quantitation of a particular drug in complicated fluids such as serum and tissue homogenates.

This article describes, in brief, radioimmunoassays for methotrexate (MTX), citrovorum factor, neocarzinostatin, and actinomycin D.

MTX Radioimmunoassay

Antibodies specific for MTX were induced in rabbits employing a covalent conjugate formed between this small drug molecule and methylated bovine serum albumin. Linkage was achieved using a water-soluble carbodiimide reagent to facilitate formation of an amide bond between the carboxyl functions of MTX and the amino groups of the protein carrier molecule.

Antisera elicited by this antigen were employed in conjunction with ^{3}H-MTX (13.7 curies/mmol) from Amersham/Searle (Arlington Heights, Ill) to produce a very sensitive radioimmunoassay (1). The system measured as little as 0.1–1 pmol (10^{-12} mols) of drug depending upon the antiserum used. Specificity studies demonstrated that naturally occurring folates are relatively poor inhibitors (fig 1). For example, compared to unlabeled MTX, an 80,000-fold molar excess of folinic acid (citrovorum factor) was required to provide commensurate displacement of the radioactive marker. Thus, it was possible to measure MTX in the presence of folates even when they were artificially elevated. This feature of the assay is essential for studying serum levels of the drug in patients receiving high-dose MTX treatment combined with citrovorum factor rescue (2). These antisera could also be used to measure aminopterin (fig 1).

In order to facilitate large-scale clinical usage of the radioimmunoassay, a nitrocellulose membrane separation technique was adapted (3). Separation of unbound from antibody-bound radiolabeled MTX was achieved by a simple and rapid filtration step (fig 2). Physiologic fluid samples containing unlabeled drug were added to the system prior to filtration and the ability to displace ^{3}H-MTX was reflected in the reduction of radioactivity bound to the filter. A standard displacement curve for unlabeled

[1] Supported in part by Public Health Service grant CA-06516 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl-, calcium salt.

Neocarzinostatin: NSC-157365; CAS reg. No. 4507-13-5.

Actinomycin D: NSC-3053. ^{3}H-actinomycin D was obtained from Amersham/Searle; unlabeled actinomycin D was obtained from Merck Sharp and Dohme.

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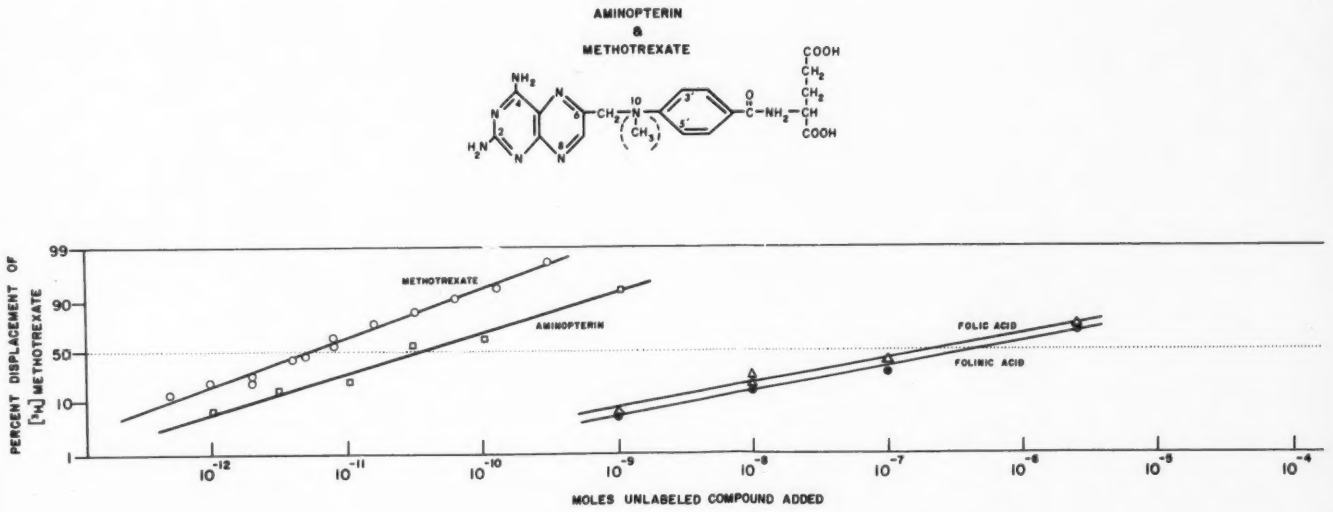

FIGURE 1.—Competitive inhibitors of the binding of ^{3}H-MTX to antibody sites. Serum (1 μl) from rabbit Ra 73-7b was used for this assay. (Adapted with permission from Raso V, and Schreiber R. Cancer Res 35:1407, 1975.)

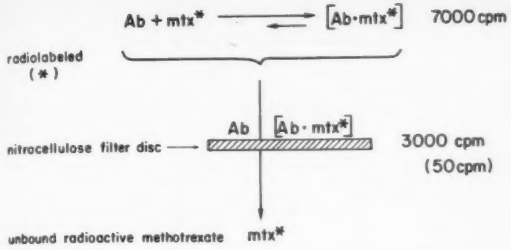

FIGURE 2.—Basic reaction mixture contained 1 μl of antiserum and 7000 cpm of ^{3}H-MTX. Typically, about 3000 cpm was retained upon the washed filter when MTX-specific serum was used, while only 50 cpm remained when nonspecific control sera were tested. Specimens containing unlabeled MTX were added to the system prior to filtration and their ability to displace ^{3}H-MTX was reflected in the reduction of radioactivity bound to the filter. Ab = antibody.

MTX served to relate the inhibition observed to the drug concentration in any specimen being assayed.

The details of this assay have been fully described (1) and it has been employed on a daily basis for > 1 year now at the Sidney Farber Cancer Institute. Its main function is to monitor serum levels in patients receiving treatment with high-dose MTX and citrovorum factor rescue. High concentrations of MTX remaining in the blood 24–72 hours after administration forewarn of impending toxicity (2).

Citrovorum Factor Radioimmunoassay

A number of microbiologic assays are available for folate determination; however, each has certain limitations which could be avoided using radioimmunoassay. Simon et al developed a radioimmunoassay for citrovorum factor utilizing antibodies directed against citrovorum factor in conjunction with ^{14}C-labeled 5-methyltetrahydrofolic acid (0.06 curie/mmol) as the radioactive marker hapten (4). This assay, however, appeared to have neither the sensitivity nor the specificity which was required for our purposes. It was reasoned that the assay could be greatly improved by employing high specific activity ^{3}H-citrovorum factor in place of the ^{14}C-tagged, 5-methyltetrahydrofolic acid. Commercially available ^{3}H-folic acid at 16 curies/mmol was formylated and reduced following a prescribed procedure (5) to yield the desired ^{3}H-5-formyltetrahydrofolic acid.

Antiserum was elicited using a conjugate of 5-formyltetrahydrofolic acid and methylated bovine serum albumin. A water-soluble carbodiimide reagent was used to form an amide linkage between the glutamic acid portion of the hapten molecule and the amino groups on the protein carrier. This chemical linkage ensures that the distinguishing features of the molecule, namely its formyl group and reduced pteridine ring, are in a readily accessible and immunodominant position (6).

The rabbit sera induced by immunization with this conjugate were shown to bind ^{3}H-citrovorum factor, and a radioimmunoassay was devised employing the same type of membrane separation technique which was utilized for MTX immunoassay. A citrovorum factor standard displacement curve showed that as little as 0.2 pmol of unlabeled compound was detectable (fig 3).

Comparisons were made between the relative ability of citrovorum factor and chemically related molecules to competitively displace ^{3}H-citrovorum factor from the antibody combining site. Specificity of the antibodies for both the 5-formyl and reduced pteridine ring was demonstrated by these displacement curves. Tetrahydrofolic acid, which lacks the 5-formyl group, displayed 300-fold lower displacing efficiency. A further 40-fold reduction was observed when a 5-methyl group replaced the formyl substituent. Molecules such as folic acid and MTX which do not possess the reduced pteridine ring were very weak inhibitors of ^{3}H-citrovorum factor binding. The 5-formyltetrahydrofolic which was used for forming the immunizing conjugate, the tritium-labeled material synthesized for the assay, and the clinical preparations which are produced by Lederle were all diastereoisomeric mixtures, the No. 6 carbon being an asymmetric position. To date it has been assumed that the radioimmunoassay does not distinguish between these two forms but this aspect of its specificity deserves further study.

This specific radioimmunoassay permits measurement of citrovorum factor during treatment of patients with high-dose MTX. The kinetics of citrovorum factor disappearance from the serum have been followed for iv and oral routes of administration, both in normal subjects and MTX-treated patients. Citrovorum factor has also been quantitated in the cerebrospinal fluid during citrovorum factor rescue of MTX. This assay has been previously reported (7) and will be described in more detail at a later date.

Neocarzinostatin Radioimmunoassay

Neocarzinostatin is an acidic, single-chain, disulfide cross-linked, polypeptide with a molecular weight of 10,700 (8). It has demonstrated cytocidal activity; however, its mode of action has not yet been fully elucidated. A preparation which was purified to homogeneity was used to immunize rabbits. The free drug when emulsified with complete

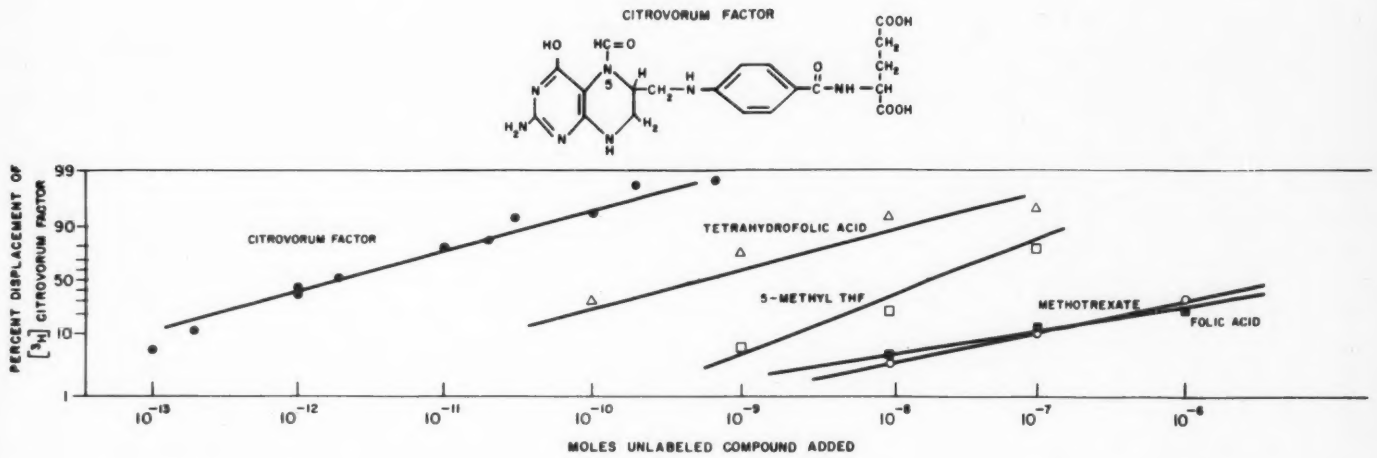

FIGURE 3.—Specificity characterization of an antiserum directed against citrovorum factor. Serum (2 μl) from rabbit Ra 74-31a was used for this assay. THF = tetrahydrofolic acid.

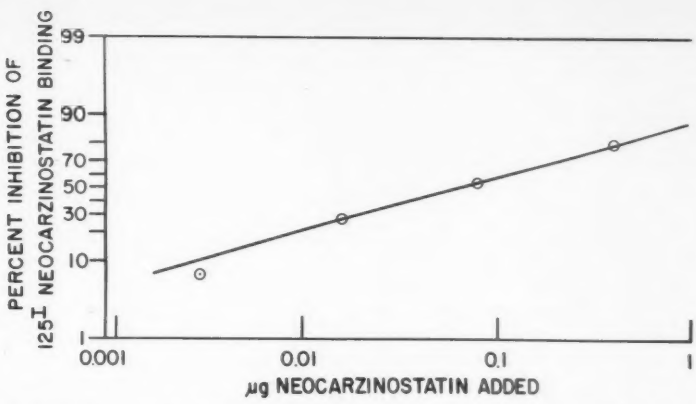

FIGURE 4.—Standard displacement curve for the neocarzinostatin radioimmunoassay. Antiserum (0.1 μl) from rabbit Ra 74-36e was used in a double antibody type assay. Increasing amounts of unlabeled drug were added to the system and the extent of displacement of ^{125}I-neocarzinostatin was determined.

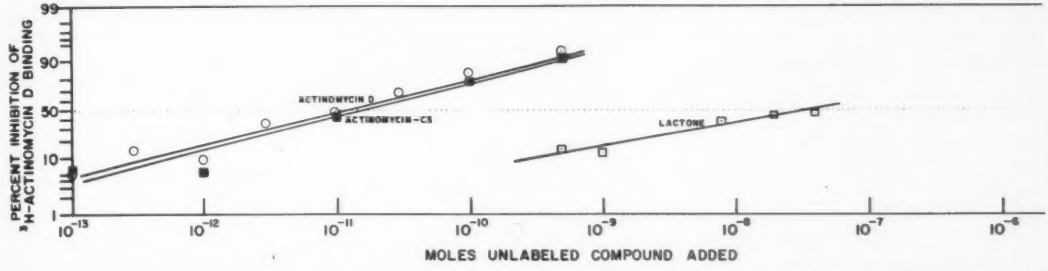

FIGURE 5.—Displacement of ^{3}H-actinomycin D from antibody by unlabeled analogs. The lactone has 1 intact and 1 hydrolized peptide ring.

Freund's adjuvant elicited an antiserum which was tested by double diffusion in gels (9). Purified neocarzinostatin and clinical preparations of the drug each gave a single line which reacted to identity when examined at optimal and at elevated concentrations. This indicated that the antiserum was monospecific and not reactive with any of the impurities present in the clinical samples.

Neocarzinostatin was reacted with ^{125}I-labeled 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccimide ester according to the method of Bolton and Hunter (10). The reaction mixture was subsequently purified by gel filtration and carboxymethyl cellulose chromatography. In this manner, the labeled neocarzinostatin was separated from both free ^{125}I ester and unreacted neocarzinostatin. This resulting labeled material was very pure, was of high specific activity, and was completely precipitable with antiserum prepared against unmodified neocarzinostatin.

With both a specific antibody and radiolabeled antigen in hand, a radioimmunoassay for neocarzinostatin was readily assembled. A fixed amount of labeled antigen was mixed with the rabbit anti-neocarzinostatin antiserum and a 30-minute incubation period was used to ensure that binding equilibrium was established. To determine the amount of antibody-bound ^{125}I-neocarzinostatin, an optimal volume of goat anti-rabbit antibody was added and after incubation and resulting precipitate was collected and washed on a glass fiber filter. The filter with its trapped precipitate was then placed in a gamma counter to quantitate the antibody-bound ^{125}I-neocarzinostatin. A standard displacement curve was established by adding known amounts of the unlabeled drug to this system and relating these to the reduction of antibody-bound radioactivity (fig 4). It can be seen from this chart that as little as 3 ng (~ 0.3 pmol) of the drug could be detected.

Actinomycin D Radioimmunoassay

The actinomycins are a group of antibiotics which possess potent cytotoxic activity by virtue of their ability to bind to DNA and prevent transcription. Since the drug is used clinically, it seemed reasonable to develop a radioimmunoassay for measuring the blood and tissue levels of actinomycin D so that

optimal therapeutic levels could be attained and excessive toxicity could be avoided.[4] A reaction was carried out with actinomycin D and bovine serum albumin in the presence of a water-soluble carbodiimide reagent. After extensive dialysis a red, insoluble complex remained and this was used to immunize rabbits. The exact form of this antigen remains uncertain. The sera of immunized rabbits were tested for their ability to bind ^{3}H-actinomycin D (12 curies/mmol). One rabbit produced a particularly potent antiserum and this was employed in a double antibody setup to produce a radioimmunoassay for actinomycin D. The assay procedure involves mixing ^{3}H-actinomycin D with rabbit anti-actinomycin D and any sample containing an unknown amount of unlabeled drug. After incubation, sheep anti-rabbit antibody was added and an immune precipitate was allowed to form. This was then collected on a glass fiber filter, washed with buffer, and dried, and the antibody-bound radioactivity was measured in a scintillation counter (Packard Instruments, Downers Grove, Ill).

A standard curve relating displacement of antibody-bound ^{3}H-actinomycin D with the amount of unlabeled compound added is shown in figure 5. Unlabeled actinomycin D can be easily detected when as little as 1 pmol has been added to the system.

Some interesting specificity characteristics were noted for this system. Actinomycin D and actinomycin C_3 were equally potent inhibitors. The molecules differ only by a substitution of an isoleucine for a valine in each polypeptide chain. This small alteration causes no apparent change in reactivity with the antibody. If, however, one of the cyclic peptides has been cleaved to form an actinomycin lactone, a dramatic loss in binding effectiveness results.

Thus, it seems that as soon as one of the cyclic peptides has broken, the integrity of the antigenic determinant has been largely destroyed.

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Detection and Microbiologic Assays of Antimetabolites [1]

Ladislav J. Hanka [2,*]

SUMMARY

The biochemical considerations applicable in the detection of new antimetabolites are very similar to those used in developing microbiologic assays for these drugs. An in vitro system for detection of antimetabolites was developed based on different sensitivities to such drugs of bacteria cultivated in complex versus completely synthetic media. A number of new antimetabolites of amino acids, vitamins, and purines or pyrimidines were isolated and microbiologic assays for these drugs were developed. As an example of the technology utilized and possibilities available, an assay for cytosine arabinoside was described. It can detect 0.5-μg/ml concentrations of this drug even in the presence of other antitumor or antibacterial antibiotics.

[Cancer Treat Rep 61:591–595, 1977]

It was our experience that the detection of new antimetabolites and microbiologic assays of such compounds become two closely intermixed projects. The reason for this was that the same set of biochemical rules had to be applied in designing cultivation media used for microbiologic assay of specific antimetabolites as was used in designing media for general detection of antimetabolites produced by microbial fermentations.

My first experience with antimetabolites was made in the late 1950s while working with a culture of a *Streptomyces* which acted in a very unusual way. It was active against experimental bacterial infection and experimental tumors in animals. Yet, it was inactive in vitro against all the common microorganisms when assayed by any of the then common methods. While investigating the possible methods for developing an in vitro assay for this unusual culture, we learned that the key to success was the utilization of a completely synthetic medium for cultivation of the assay organism. Later, we learned that the antibiotic we were dealing with was psicofuranine (angustmycin A). We then found that its antimicrobial effect was readily antagonized by minute concentrations of guanine or its derivatives (1). Since the commonly used rich media (nutrient agar, BHI agar, etc) contain such materials, no in vitro activity was observed when such media were used.

A similar experience was made a few years later while working with the culture producing 5-azacytidine (2).

The basic observation was similar: no in vitro activity when the test organism was grown on a complex medium but strong in vitro activity when the same organism was cultivated in a synthetic medium. In vitro activity was antagonized by supplementing the synthetic medium with cytidine. We have tested a few known antimetabolites and the results of this study are shown in table 1. All the antimetabolites inhibited the growth of the susceptible microorganism more on the synthetic medium than on a complex medium. This was in sharp contrast with results recorded with antibiotics such as chloramphenicol, streptomycin, neomycin, etc, that generally inhibited a microorganism to a comparable degree regardless of the medium used.

It occurred to us that this observation could be put to practical use and, as a result, a specific detection system for antimetabolites was designed and first described in 1967 (3). We have used it for several years to detect antimetabolites produced by fermentation of soil microorganisms. We have isolated several antimetabolites of different amino acids (4,5), three different antimetabolites of biotin (6,7), and 5-azacytidine (2,8). The technique has

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TABLE 1.—Inhibition of *Escherichia coli* (*E. coli*) and *B. subtilis* grown in undefined and defined media (*from* ref 3)

Drug	Concentration (μg/ml)	Test organism	Zone of inhibition (mm) on—	
			Nutrient agar	Synthetic agar
5-FUDR (NSC-27640)	5	*E. coli*	0	22
6-Azauracil (NSC-3425)	50	*E. coli*	0	24
6-Thioguanine (NSC-752)	100	*E. coli*	0	33
5-Azacytidine (NSC-102816)	10	*E. coli*	0	25
Methotrexate (NSC-740)	5	*B. subtilis*	0	40
6-Mercaptopurine (NSC-755)	100	*B. subtilis*	0	26

TABLE 2.—Inhibition by several fermentation liquors of *B. subtilis* or *E. coli* grown in 2 different media (*from* ref 3)

Sample No.	Microorganism	Zone of inhibition (mm) on—	
		Nutrient agar	Synthetic agar
1	*B. subtilis*	0	30
1	*E. coli*	0	27
2	*B. subtilis*	0	0
2	*E. coli*	0	29
3	*B. subtilis*	23	25
3	*E. coli*	25	20

gained wide acceptance; for example, it has been used successfully by investigators at Hoffmann-La Roche Inc, Nutley, NJ, and by Professor G. Gause at the Soviet Academy of Medical Sciences, Moscow.

The screening technique is rather simple and suitable for evaluation of large numbers of samples. In three successive experiments, it is possible to detect potential antimetabolites and then to learn something of their mode of action (tables 1–3).

In table 2, both samples 1 and 2 had in vitro activity typical for an antimetabolite—no activity on complex medium and strong in vitro activity on synthetic agar. Sample 3 acted in a manner typical for most antibacterial drugs and was of no further interest. Samples 1 and 2 were further tested to determine the broad area of their metabolic involvement and the results of this experiment are presented in table 3.

In table 3 the inhibition of the test organism by sample 1 was prevented by amino acids. It was reasonable to assume that the drug produced by this culture interfered with the metabolism of one (or more) of the amino acids. Sample 2 apparently was involved in the metabolism of nucleic acids since it was antagonized by their components, purines and pyrimidines. In another simple experiment we were able to determine which specific compound of the mixture was involved. The data in table 4 indicate that the drug produced by this culture was apparently an antimetabolite of cytidine (or uridine), since cytidine completely reversed its in vitro effect.

Later, these conclusions were proven to be correct since sample 2 was the culture producing 5-azacytidine, a drug of considerable interest today.

To look at these results from another angle, it is possible to modify this screen so that it will be detecting almost specifically antimetabolites of amino acids. In this case the synthetic medium will be further supplemented with all the common vitamins as well as purines and pyrimidines, but no amino acids.

TABLE 3.—Inhibition by 2 fermentation liquors of *E. coli* grown in different media (zones of inhibition [mm]) (*from* ref 3)

Sample No.	Nutrient agar	Synthetic agar				
		No supplements	Further supplemented with—			
			Combined amino acids	Combined vitamins	Combined purines and pyrimidines	Krebs cycle intermediates
1	0	27	0	24	26	26
2	0	29	27	30	0	31

TABLE 4.—Reversal by different metabolites of the inhibition of *E. coli* by the fermentation liquor 2 (*from* ref 3)

Addition* to synthetic agar	Zone of inhibition in mm (around a 13-mm disc)
Control, no addition	31
Adenosine	28
Guanosine	30
Inosine	31
Xanthosine	31
Uridine	17 (hazy zone)
Cytidine	0
Thymidine	30

*All additions at 50 μg/ml.

Microbiologic Assay for Antimetabolites

The problems facing us in this area can be broadly divided in two categories.

1. Problems inherent to the development of any microbiologic assay.
2. Problems peculiar to the chemical structure and biochemical behavior of the specific antimetabolite.

To tackle the problems of the first category it is necessary to have a good basic understanding of microbial growth, nutrition, and inhibitions. The selection of the most suitable microorganism is of the highest importance. It is not necessary to limit ourselves to the commonly utilized bacteria. It is desirable to consider also yeasts, protozoa, etc. Unusual mutants have been uniquely useful at times. Cytosine arabinoside was totally inactive in in vitro bacterial systems until Pittillo and Hunt (9) included an actinobolin-resistant mutant of *Streptococcus faecalis* (*S. faecalis*) in several hundred of the other microorganisms evaluated for their possible sensitivity towards that drug.

In the microbiologic assays for antimetabolites, there is one additional requirement that has to be taken into consideration: the medium used for the cultivation of the test organism must not contain a compound(s) potentially capable of antagonizing the drug tested. A specific example will illustrate this point. A completely synthetic medium used for one strain of *Bacillus subtilis* (*B. subtilis*) has the following composition: $Na_2HPO_4 \cdot 7H_2O$, 1.7 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4$, 0.1 g; glucose, 2.0 g; metallic ions stock solution (3), 1 ml; and distilled water, 1 liter. Obviously, this system is potentially suitable for assay of any antimetabolite. On the other hand, we have worked with other members of the genus *Bacillus* that required addition of some amino acids to the growth media. Such microorganisms would not be suitable for assay of antimetabolites of these amino acids. Equally, an organism requiring purines or pyrimidines for growth cannot be used for assays of antimetabolites of nucleic acid precursors.

Specific Assays

A number of assays for different antimetabolites have been developed in our laboratories. I present one which suggests how specific and versatile the microbiologic assay can be.

Cytosine arabinoside is a drug used primarily in the treatment of leukemia (10). In the late 1960s several investigators tried to develop a microbiologic assay for this drug which at first appeared to be completely inactive in vitro. Pittillo and Hunt (9) reported the first activity against an actinobolin-resistant mutant of *S. faecalis*. The sensitivity of their assay (3 μg/ml) was inadequate for our needs, however. We were able to increase its sensitivity ~25 times mainly by incorporating low levels of tetrahydrouridine into the assay medium (11). This drug is a strong inhibitor of the pyrimidine nucleoside deaminase which inactivates cytosine arabinoside by converting it to a biologically inactive metabolite, uracil arabinoside. Leukemia is commonly treated using a combination of several antitumor drugs. Furthermore, some of the patients may be treated simultaneously with antibacterial antibiotics. Thus, it appeared that it would be desirable to develop modifications of the assay that would make it possible to measure the levels of cytosine arabinoside in materials containing other antitumor and/or antibacterial drugs (12).

Thus, we have tested the sensitivity of *S. faecalis* towards 13 clinically useful antitumor drugs. As can be seen in table 5, among the drugs most frequently

TABLE 5.—Inhibition of *S. faecalis*/actinobolin by antitumor drugs (*from* ref 12)

Drug*	Zone of inhibition in mm (13-mm disc)
Mechlorethamine (NSC-762)	—
6-Mercaptopurine (NSC-755)	—
Methotrexate (NSC-740)	41, hazy
6-Thioguanine (NSC-752)	—
Daunorubicin (NSC-82151)	—
Hydroxyurea (NSC-32065)	—
L-Asparaginase (NSC-109229)	—
Vinblastine (NSC-49842)	—
Vincristine (NSC-67574)	—
5-Fluorouracil (NSC-19893)	—
Prednisone (NSC-10023)	—
Actinomycin D (NSC-3053)	32
BCNU (NSC-409962)	0
Cytosine arabinoside (NSC-63878), 1.25 μg/ml	26

*Volumes: 0.08 ml of 50-μg/ml solutions used per disc.

TABLE 6.—Inhibition by cytosine arabinoside of *S. faecalis*/actinobolin grown in the presence of various levels of deoxycytidine (*from* ref 12)

Concentration of cytosine arabinoside (μg/ml)	Concentration of deoxycytidine (μg/ml)					
	0	1.25	2.5	5.0	10.0	20.0
1.25	25.8*	—	—	—	—	—
2.5	28.8	—	—	—	—	—
5.0	31.5	18	—	—	—	—
10.0	34.3	22	16.3, hazy	—	—	—

*Zones of inhibition (mm) around a 13-mm paper disc.

TABLE 7.—Inhibition of *S. faecalis*/actinobolin by antibiotics (*from* ref 12)

Sensi-Discs	Concentration of deoxycytidine (μg/ml)			
	0	1	10	100
Penicillin 2	9*	9	12	12
Ampicillin 10	30	29	31	31
Erythromycin 2	21	20	19	17
Lincomycin 2	21	21	19	21
Cephalothin 30	21	21	21	21
Tetracycline 5	26	28	25	30
Chloramphenicol	24	24	23	23
Neomycin 30	0	0	0	0
d-Streptomycin 2	0	0	0	0
Polymyxin B 50	0	0	0	0
Triple sulfa 0.25	0	0	0	0
Nystatin 100	0	0	0	0

*Zones of inhibition (mm) around a 6.5-mm paper disc.

used in the treatment of leukemia, only methotrexate was inhibitory to the test organism. Such activity can be completely prevented by incorporating folinic acid into assay media. Folinic acid will completely reverse the activity of methotrexate, yet has no effect on in vitro activity of cytosine arabinoside. Mehta et al (13) have recently described a more sensitive assay method using a methotrexate- and 6-mercaptopurine-resistant mutant.

The assay organism (*S. faecalis*) is also sensitive to a number of common antibacterial antibiotics. It is possible to assay for such drugs in media supplemented with deoxycytidine. As can be seen in table 6, deoxycytidine antagonized effectively the inhibition of *S. faecalis* by cytosine arabinoside. On the other hand, it had no effect on the in vitro activity of the common antibacterial antibiotics, as can be seen from table 7.

To eliminate the interference of antibacterial antibiotics in the assay, several special techniques can be used. For instance, the interference by penicillin and ampicillin can be eliminated by treatment of the samples with penicillinase. The penicillinase has no effect on the activity of cytosine arabinoside. It should be possible to develop mutants of *S. faecalis*/actinobolin resistant to one or several antibacterial drugs; however, in cases of antibiotics where no such special approach is feasible, chromatography followed by bioautography against *S. faecalis*/actinobolin must be used.

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Microbiologic Assays of Cancer Chemotherapeutic Agents[1]

Bipin M. Mehta* and Dorris J. Hutchison[2,3]

SUMMARY

Among various assay methods available for the study of drug distribution kinetics in cancer chemotherapy, a method that determines the drug in its active form is the most desirable. The microbiologic assay method, with its rapid, simple, and inexpensive procedures, fulfills such a requirement. Besides the two commonly used chemotherapeutic agents, methotrexate and cytosine arabinoside, microbiologic assays are available for other chemotherapeutic agents, such as 6-mercaptopurine, 5-fluorouracil, actinomycin D, adriamycin, and daunorubicin. Assays have been developed for citrovorum factor and 5-methyltetrahydrofolate in the presence of high levels of methotrexate. With the introduction of combination chemotherapy, microbiologic assay methods using resistant mutants, sensitive to a single drug but resistant (either due to mutation or due to culture conditions) to all other drugs involved in the combination, could well be the most efficient for the study of distribution kinetics. Several such assay methods employed in our laboratory are discussed.

[Cancer Treat Rep 61:597–601, 1977]

The study of drug distribution kinetics in cancer chemotherapy has utilized various methods: spectrophotometric, colorimetric, isotopic, chromatographic, enzymatic, biologic (microbiologic and tissue culture), and radioimmunoassay (1–7). Since it is desirable to have a method which determines the drug in its active form, biologic assays, and especially microbiologic assays, are invaluable because of their simplicity, rapidity, and low cost. Microbiologic assays have been developed for a number of antitumor agents (3,8–11) and are based on the specific knowledge of the microorganism that is sensitive to inhibition by the antitumor compounds. A microbiologic assay (12), based on the specific growth requirement of a microorganism, has evolved as a direct result of the recently developed treatment of childhood acute leukemia and osteogenic sarcoma utilizing a high-dose methotrexate-citrovorum factor rescue regimen (13,14). We describe here some of the microbiologic assays, either growth inhibitory or growth promoting, which have been developed or modified for the study of pharmacokinetics of certain cancer chemotherapeutic agents in tissues and body fluids. With the introduction of combination chemotherapy, the problem of distribution kinetics of various chemotherapeutic agents involved becomes more complicated. However, microbiologic assay techniques, using resistant mutants sensitive to a single chemotherapeutic agent but resistant to the rest of the agents involved in the combination, provide the easy solution to such a problem.

MATERIALS AND METHODS

Determination of Methotrexate

The microorganism used for the determination of methotrexate is a wild type strain of *Streptococcus faecium* var. *durans* which is designated SF/0. The strain SF/0 was maintained, as described by Hutchison (15), on the folic-acid assay medium of Flynn et al (16) containing 1 ng/ml of folic acid. Inoculum for

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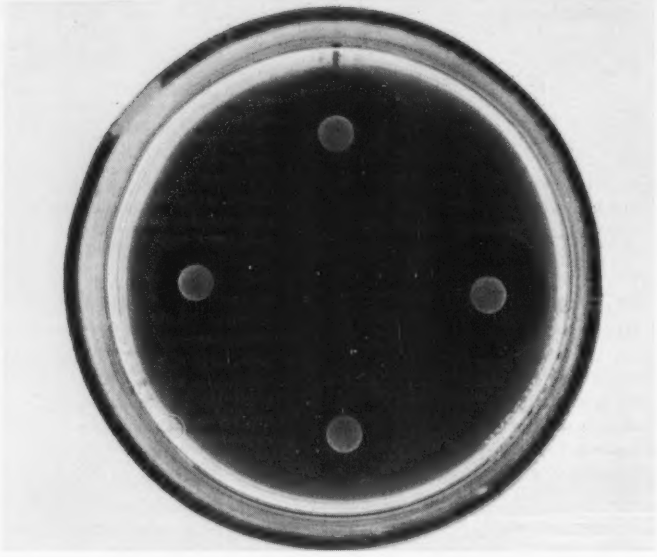

FIGURE 1.—Growth inhibition of SF/0 by methotrexate. Zones of inhibition with standard solutions of the drug containing (clockwise) 50, 30, 10, and 5 ng/ml in 0.85% saline; 0.02 ml applied to each of the respective discs (6.35-mm diameter). Assay medium was that described by Flynn and 0.5 ng/ml of folic acid.

the assay was prepared as follows. SF/0 was grown overnight for 18 hours at 37°C in the liquid folic-acid assay medium of Flynn et al (16) supplemented with folic acid (1 ng/ml). The culture was then washed twice with and resuspended in 0.85% saline to an optical density of 0.2 at 600 nm (approximately 6×10^7 cells/ml) as measured in a Bausch and Lomb Spectronic 20 spectrophotometer; 2.5 ml of the cell suspension was used as inoculum for 200-ml folic-acid assay medium of Flynn et al (16) containing 1.5% agar and supplemented with 0.5 ng/ml of folic acid. Flat bottom glass plates containing 10 ml each of the seeded agar medium were prepared and stored in the cold for at least 3–4 hours at 4°C prior to use for the assay. The solutions for the standard curve were made with a purified sample of methotrexate (Lederle Laboratories, Division of American Cyanamid Co, Pearl River, NY) in saline at concentrations of 5, 10, 30, and 50 ng/ml. Paper discs (Schleicher and Schuell, No. 740-E) with a 6.35-mm diameter were soaked with 20 μl of the standard methotrexate solutions and placed on the plates after drying in the incubator at 37°C for at least 1–1½ hours. The samples of body fluids such as serum, plasma, tissue homogenates, and cerebrospinal fluid (CSF) were also applied to the discs similarly after proper dilutions without any prior treatment or sterilization. The plates were then incubated for 18 hours at 37°C. Zones of inhibition (fig 1) were measured in a zone reader (Fisher Lilly Antibiotic Zone Reader, Fisher Scientific Co, Pittsburgh, Pa).

The standard curve was plotted as an average of five zone diameters in millimeters against the logarithm of the known concentrations (fig 2). The concentrations of the unknown samples were then determined from the standard curve. The statistical variation in the methotrexate determination using this method was within 7.8% ± 2.6% (standard deviation [SD]). The recovery of methotrexate from body fluids such as serum, plasma, and CSF (both ventricular and lumbar) was 97.8% ± 4.4% (SD). The lower limit of sensitivity obtained by this method with tissues and body fluid samples was 5×10^{-9} M. The use of larger paper discs (13-mm diameter) increased the sensitivity of the assay by fivefold which enabled us to detect 10 pg (2×10^{-11} mols) of methotrexate. The method described here is a more sensitive modification of the earlier method described by Burchenal et al (17) and has been extensively used in studies on pharmacokinetics (18,19).

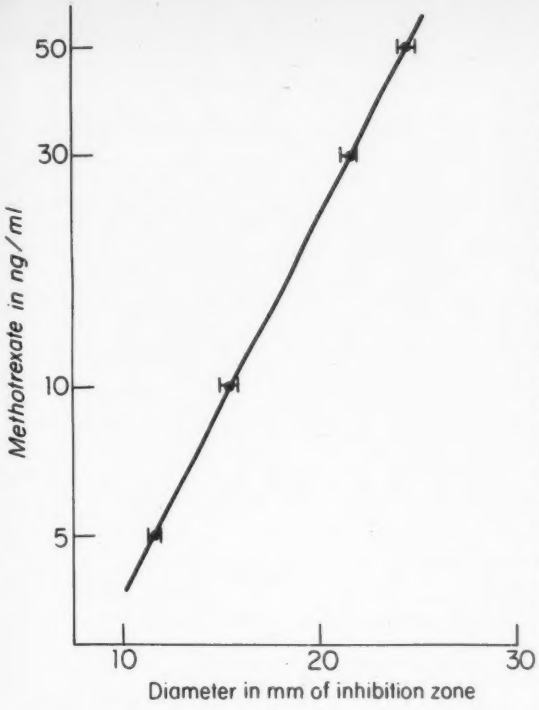

FIGURE 2.—Standard curve of growth inhibition with methotrexate; bars represent SD of the mean values.

Determination of Cytosine Arabinoside

Another invaluable microbiologic assay which was developed in our laboratory is for cytosine arabinoside which relies on a strain of *S. faecium* var. *durans* resistant to methotrexate and 6-mercaptopurine (11). This strain was first described by Hutchison (15) and is designated SF/A/MP. The strain SF/A/MP was found to be more sensitive to cytosine arabinoside than the commonly used actinobolin-resistant strain of *S. faecalis* ATCC 8043. The importance of this assay for cytosine arabinoside using SF/A/MP (11) is that it determines the concentration of cytosine arabinoside in the presence of methotrexate, 6-mercaptopurine, and 6-thioguanine.

Determination of 6-Mercaptopurine, 6-Thioguanine, and 5-Fluorouracil

Microbiologic assays are available for a few of the commonly used cancer chemotherapeutic agents in the clinic (table 1) in addition to methotrexate and cytosine arabinoside. The organism for the assay of

TABLE 1.—Microbiologic assays

Agent	Microorganism	Sensitivity (μg/ml)
Methotrexate	SF/0	0.0025
Cytosine arabinoside	SF/A/MP	0.05
6-Mercaptopurine	SF/0	0.5
6-Thioguanine	SF/0	0.5
5-Fluorouracil	SF/A	0.01
Citrovorum factor	PC/A	0.001
Folic acid	SF/A	0.005
5-Methyltetrahydrofolate	LC/A	0.005

6-mercaptopurine, 6-thioguanine, and 5-fluorouracil may be SF/0 or a methotrexate-resistant strain which is designated SF/A, depending on whether one wants to determine the concentration of the drug in question alone or in the presence of methotrexate. The isolation and characterization of all the strains of *S. faecium* var. *durans* were carried out by Hutchison (15), and the strains were maintained accordingly. Although the microbiologic assay procedure for most of the inhibitory agents was the same as described for methotrexate, the concentrations for the standard curve and the composition of the medium were different for each agent.

Although the same organism is used for the assay of 6-mercaptopurine, 6-thioguanine, and 5-fluorouracil, a manipulation of the assay medium is necessary for achieving optimal response. The folic-acid assay medium of Flynn et al (16), a well-defined medium, can be altered as desired. Thus, the assay of 5-fluorouracil described by Clarkson et al (20) used the assay medium that was a uracil-free modification of the folic-acid assay medium of Flynn et al (16) supplemented with 3 ng/ml of folic acid. Similarly, in order to assay for 6-mercaptopurine and 6-thioguanine, one uses a medium that is a purine-free modification of the folic-acid assay medium of Flynn et al (16) supplemented with 3–5 ng/ml of folic acid. The assay organism, SF/0, is resistant to cytosine arabinoside in the presence as well as in the absence of purines, hence, 5-fluorouracil, 6-mercaptopurine, and 6-thioguanine can be assayed in the presence of cytosine arabinoside.

All of the organisms listed in table 1 are resistant to actinomycin D, adriamycin, daunorubicin, and some of the alkylating agents at relatively high concentrations. Thus, it is possible to assay all of these drugs in the presence of large amounts of actinomycin D, adriamycin, daunorubicin, vincristine, prednisone, CCNU, and BCNU.

Determination of Citrovorum Factor, 5-Methyltetrahydrofolate, and Folic Acid

In addition to the assays described above, which are based on growth inhibition, table 1 also lists

assays that are based on the growth-promoting characteristics of the compound. Thus, the assays for citrovorum factor, folic acid, and 5-methyltetrahydrofolate are based on the determination of zones of growth rather than zones of growth inhibition. The microbiologic assay for citrovorum factor in the presence of methotrexate (12), using a methotrexate-resistant strain of *Pediococcus cerevisiae* (PC/A), is used in our laboratory for routine determinations of citrovorum factor in the serum and other body fluids and tumors of patients treated with the high-dose methotrexate-citrovorum factor rescue regimen. Hutchison and Burchenal (21) isolated this methotrexate-resistant strain of *Leuconostoc citrovorum* ATCC 8081, which was later identified as PC/A (22).

Since most of the citrovorum factor is rapidly converted to 5-methyltetrahydrofolate following administration (23), determination of both 5-methyltetrahydrofolate and citrovorum factor in the samples of biologic fluids might be a meaningful approach to the understanding of the rescue regimen.

We have isolated a strain of *Lactobacillus casei* resistant to methotrexate (LC/A) that is used in a differential assay for 5-methyltetrahydrofolate. A disc assay similar to the citrovorum factor assay (12) has been designed using LC/A to determine 5-methyltetrahydrofolate on the basis of the differential microbiologic assay system established by Bird et al (24). Since LC/A determines the total folates, SF/A determines the total folates, except 5-methyltetrahydrofolate, and PC/A determines citrovorum factor only, the quantitation of both 5-methyltetrahydrofolate and citrovorum factor can very easily be achieved by differential assays. This method has been used in our laboratory successfully for a study determining the distribution of both 5-methyltetrahydrofolate and citrovorum factor following oral and intramuscular administration of citrovorum factor (unpublished data).

DISCUSSION

The microbiologic assays developed in our laboratory for the estimation of levels of certain cancer chemotherapeutic agents in biologic fluids are listed in table 1. However, in the present era of combination chemotherapy, a need for the determination of some of these agents in the presence of other more common chemotherapeutic agents is well recognized. Microbiologic assays with the availability of wide varieties of the resistant mutants make it possible to specifically assay some of these agents in the presence of other agents and in combination. Table 2 summarizes such presently available assay methods which we have for use (18,19) in pharmacokinetic studies following combination chemotherapy.

Determination of methotrexate in the presence of high levels of citrovorum factor does pose a problem because of the reversal of the inhibitory action of methotrexate by citrovorum factor. For such an interference to occur in our assay system, the concentration of citrovorum factor has to be equimolar or greater than that of methotrexate.

Since most of the citrovorum factor is rapidly converted to 5-methyltetrahydrofolate which does not interfere with the assay system, we have a minimal problem with samples of patients treated with the high-dose methotrexate-citrovorum factor rescue regimen. In the few cases where the citrovorum factor level is either equimolar or greater than that of methotrexate, we follow the dihydrofolate reductase assay method described by Sirotnak et al (25).

TABLE 2.—Available microbiologic assays of antimetabolites and metabolites in the presence of other agents used in chemotherapy

Assay for—	Other agents present*								
	Methotrexate	Cytosine arabinoside	6-Mercaptopurine	6-Thioguanine	Actinomycin D	Daunorubicin	Citrovorum factor	Folic acid	5-Methyltetrahydrofolate
Methotrexate		+	+	+	+	+	?	+	+
Cytosine arabinoside	+		+	+	+	+	+	+	+
6-Mercaptopurine	+	+			+	+	+	+	+
6-Thioguanine	+	+			+	+	+	+	+
5-Fluorouracil	+	+	+	+	+	+	+	+	+
Citrovorum factor	+	+	+	+	+	+		+	+
Folic acid	+	+	+	+	+	+	+		+
5-Methyltetrahydrofolate	+	+	+	+	+	+	+	+	

*+ = sensitivity of assay not altered by presence of the other agent; ? = sensitivity of assay questionable in presence of excess citrovorum factor.

Microbiologic assay methods for actinomycin D,[4] adriamycin (26), and daunorubicin (27) have been described; however, these drugs are administered to patients in such quantities that even assuming rapid and uniform distribution, the maximum theoretically obtainable concentrations of these drugs in the body fluids are below the sensitivity of the assay. Thus, the need for more sensitive assay methods for these compounds is urgent.

Microbiologic assays are thus sufficiently sensitive, rapid, and specific for the determination of certain cancer chemotherapeutic agents described here. With a thorough knowledge of the assay organism and the mode of action of the agent it assays, one can measure the concentration of agents which are of major importance in cancer chemotherapy.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl}-, calcium salt

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Folic acid: NSC-3073; glutamic acid, *N*-[*p*-[[(2-amino-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl}-)

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

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Kinetics of Elimination of Methotrexate From the Cerebrospinal Fluid Space of Monkeys After Ventriculolumbar Perfusion [1,2]

Philip C. Merker,[3,*] Bipin M. Mehta,[4] Margaret L. Cantor,[3] and Dorris J. Hutchison [4,5]

SUMMARY

The kinetics of elimination of methotrexate from the cerebrospinal fluid (CSF) space of rhesus monkeys after ventriculolumbar (VL) perfusion were studied for the purpose of determining the suitability of the monkey as a preclinical model for CSF drug elimination experiments. Rhesus monkeys were perfused through the left lateral VL subarachnoid space with Elliott's B solution containing 0.3 mg/ml (6.6×10^{-4} M) of methotrexate. Perfusions were conducted at an average inflow (Ci) rate of 0.128 ml/minute for about 200 minutes. Samples of CSF were collected from the lumbar subarachnoid space and venous blood at selected time intervals following the perfusion for a 24-hour period, and each sample was assayed microbiologically for methotrexate. The concentration of methotrexate in lumbar outflow (Co) samples during drug perfusion reached a plateau in about 60 minutes; during the plateau period, methotrexate concentration in Co samples ranged from 2.5 to 5.2×10^{-4} M, and the average ratio of Co/Ci during the plateau phase was 0.68 ± 0.16. The elimination of methotrexate from the CSF space occurred in two phases: (*a*) the first (rapid) phase occurred during the first 3 hours when the concentration of drug in the CSF declined from about 5×10^{-4} to about 5×10^{-5} M (the half-life during this first phase was calculated to be about 45 minutes), and (*b*) the second (slow) phase occurred between the 3rd and 24th hours when drug concentration declined to an average final value of 5×10^{-7} M (the half-life during this period was calculated to be about 3 hours). Concentrations of methotrexate $\geq 10^{-6}$ M persisted in the CSF for about 16–20 hours, and the concentration of methotrexate at 24 hours following perfusion was at least 10^{-7} M. The kinetics of elimination of methotrexate following VL perfusion were found to be similar to those reported in the literature for man following intrathecal and intraventricular administration. Accordingly, the monkey may be a useful model in experiments designed to study the kinetics of drug movement from the CSF space.

[Cancer Treat Rep 61:603–611, 1977]

Pharmacokinetic studies in patients treated intrathecally with methotrexate suggest that frequent monitoring of methotrexate in cerebrospinal fluid (CSF) and appropriate adjustment of drug dosage or treatment interval may be helpful in preventing neurotoxicity (1). Young et al (2) and Shapiro et al (3) have studied the pharmacokinetics of methotrexate in patients given the drug intraventricularly, and their data indicate that the intraventricular route of administration may produce more reliable drug distribution within the CSF space compared to the intrathecal route.

The CSF half-life for methotrexate injected via the intraventricular route is reported to be 3 hours

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Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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[5]We would like to acknowledge the encouragement of Dr. Michael D. Walker during the course of these studies.

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(2,3). Rubin et al (4) have reported a half-life for methotrexate of 3.6 hours in man following a 4-hour lateral ventricle to lumbar subarachnoid space perfusion, and Bleyer et al (1) have shown that the half-life for methotrexate injected intrathecally is 12–18 hours. Accordingly, these clinical studies have demonstrated that the biologic half-life of methotrexate in the CSF space of man is > 1 hour. Ventriculolumbar (VL) perfusion experiments were therefore done in monkeys to measure the biologic half-life of methotrexate in the CSF space following perfusion for the purpose of determining the usefulness of this animal model in planning studies designed to inject, infuse, or perfuse drugs into the CSF spaces of man.

MATERIALS AND METHODS

The detailed methods and procedures used to perfuse drugs through the CSF spaces of monkeys have been previously described (5). For the purposes of this work, a brief description is given.

Animals

Two male rhesus monkeys (*Macaca mulatta*) were used in these pilot studies; the starting weights were 3.2 and 4.4 kg. The monkeys were maintained in individual cages and given free access to water and 150–200 g/day of Purina food (Ralston Purina Co, St. Louis, Mo).

Cannulation Sites

The cannulation site for the left lateral ventricle was at the anteroposterior plane of the anterior commissure and foramen of Monro, A.17.0, L1.0–2.0, H12.0, according to the atlas of Snider and Lee (6).

A 0.020 × 0.037-inch tubing (Silastic, Dow-Corning, Midland, Mich) was threaded into the lumbar subarachnoid space at the level of L4–5 for the collection of lumbar outflow (Co).

Perfusion

During VL perfusion, the animals were placed in the prone position under light anesthesia induced by either an initial phencyclidine (Sernylan, Parke, Davis and Co, Detroit, Mich) dose of 1 mg/kg im followed by maintenance doses equivalent to about 0.3 mg/kg/hour or pentobarbital sodium (Nembutal, Abbott Laboratories, N Chicago, Ill), at an initial dose of 2 mg/kg iv supplemented with 0.6 mg/kg/hour iv.

A Harvard syringe pump was used to perfuse the VL space for 190–200 minutes at a calibrated flow rate of 129–130 μl/minute, about three times the normal rate of CSF formation. A Statham pressure transducer (model P23Db, Statham Instruments, Oxnard, Calif), which was set at the height of the external auditory meatus as reference, was used to monitor CSF pressure continuously. The average height of the orifice of the Co tubing relative to the auditory meatus was adjusted to about −8 cm.

Prior to perfusing with drug, monkeys were given at least two control perfusions with Elliott's B solution containing trace amounts of radiolabeled inulin in order to make certain that they were good candidates for drug perfusion experiments.

Perfusion Fluid

The perfusion fluid was Elliott's B solution, originally described by Elliott and Jasper (7), and was manufactured sterile and pyrogen-free by Baxter Laboratories, Morton Grove, Ill. In all cases [methoxy-^{3}H]inulin, obtained as a sterile aqueous solution (New England Nuclear Corp, Boston, Mass, Lot Nos. 799–055, 761–162), was added to the Elliott's solution in a concentration of 5 millimicrocuries [^{3}H]inulin/ml perfusion fluid; at the specific activity supplied, this was equivalent to approximately 2 μg/ml perfusion fluid. In the case of control perfusions, the pH was adjusted to 7.3–7.4 and the solution was passed through a 0.22μ-Millipore filter (Millipore, New Bedford, Mass). Aliquots of lumbar Co, which were collected at appropriate time intervals, were counted in a scintillation counter (Mark I, model 6860, Nuclear-Chicago, Des Plaines, Ill). To reduce sources of counting error, samples were treated with 0.1 ml 1% sodium periodate in 1 M formic acid. The radiolabeled inulin served as a marker substance for measuring CSF formation rate which was calculated as described by Heisey et al (8) using the equations of Pappenheimer et al (9).

Methotrexate

Methotrexate, obtained from clinical-dose vials containing 50 mg of drug and 0.86 mg of NaCl with no preservatives added (supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md; Lot No. BV-73-253), was dissolved in Elliott's B solution to yield a final drug concentration of 0.3 mg/ml (6.6×10^{-4} M).

Blood Sampling

Blood samples were collected via a Venocath-18 (Abbott Laboratories, Lot No. 4718). At appropriate time intervals which matched the collection of lumbar Co samples, 1.0–1.5 ml of blood was withdrawn

into individual 2.5-ml plastic syringes; the blood samples were then added to vacutainers containing K_3EDTA (0.008 mg) as an anticoagulant. Plasma samples were assayed for methotrexate using microbiologic procedures described below.

Methotrexate Assay

The concentrations of methotrexate in plasma and lumbar Co samples were determined microbiologically using a disc assay technique (10). A standard curve was prepared using highly purified methotrexate (obtained from Lederle Laboratories, Pearl River, NY) in solutions of 1, 2, 6, and 10×10^{-8} M concentrations applied to filter paper discs (Schleicher and Schuell, Keene, NH No. 740-E) of 6.35-mm diameter each in 0.02-ml quantities. The samples to be assayed were applied similarly to filter paper discs and all the discs were dried for 1–1½ hours. Plates used in the assay contained Flynn medium (11), 0.5 ng/ml folic acid, and 1.5% agar, and were seeded with *Streptococcus faecium* var. *durans* freshly prepared weekly. The dried discs containing standard methotrexate concentrations and unknown samples were placed on the solidified agar plates which were then incubated for 18 hours at 37°C. Clear zones of inhibition appeared on the plates around the filter paper discs. A standard curve was prepared plotting the diameter of the zone of inhibition (measured by the Fisher-Lilly Antibiotic Zone Reader, Fisher Scientific Co, Pittsburgh, Pa) against the log of the concentration of the known standard samples of methotrexate. The concentration of the unknown samples, assayed in triplicate, was determined from the standard curve.

Calculation of Quantity of Drug Perfused

The total quantity of drug introduced into the CSF was determined by taking into account perfusate concentration, inflow (Ci) rate, and perfusion duration.

RESULTS

Non-Drug Elliott's B Perfusions Prior to Methotrexate Perfusions

Data from non-drug Elliott's B perfusions showed that both monkeys were acceptable candidates for drug perfusion experiments: vital signs were relatively stable; lumbar Co rates were within acceptable limits relative to Ci rates; and perfusion pressures, inulin clearance, and CSF rates of formation were also within the expected range for monkeys perfused under these experimental conditions (tables 1 and 2). The concentration of radiolabeled inulin equivalents in lumbar Co reached a plateau value at about 60 minutes after the start of perfusion, and the ratio of radiolabeled inulin equivalents in lumbar Co to ventricular Ci concentration (Co/Ci) during the final 60 minutes of the plateau phase ranged from 0.65 to 0.80 (table 3).

TABLE 1.—Vital signs of monkeys during VL subarachnoid space perfusion with methotrexate*

Monkey No./sex	Perfusion	Interval between perfusion (days)	Rates (ml/min)		Duration of perfusion (mins)	Amount of drug perfused (mg)	Body weight (kg)	Body temperature (°F)		Heart rate (per min)		Respiration (per min)	
			Ci	Co				Initial	Final	Initial	Final	Initial	Final
156-KK/M	Elliott's B	—	0.130	0.089	99	—	4.4	98.8	—	138	144	30	30
	Elliott's B	8	0.128	0.168	194	—	4.4	100.0	99.6	150	162	27	39
	Methotrexate, experiment 1	5	0.128	0.167	198	7.6	4.2	100.3	99.8	138	138	24	30
	Elliott's B	15	0.128	0.142	198	—	4.6	—	—	180	114	30	15
	Methotrexate, experiment 2	27	0.128	0.082	189	7.3	5.1	101.2	100.4	138	108	39	36
	Elliott's B	22	0.127	0.127	200	—	4.9	100.2	101.2	120	114	24	39
157-KK/M	Elliott's B	—	0.128	0.090	193	—	3.2	100.6	100.2	168	150	30	33
	Elliott's B	10	0.128	0.113	190	—	3.7	99.2	99.6	132	132	30	42
	Methotrexate, experiment 1	6	0.128	0.154	193	7.4	3.7	101.0	101.6	156	180	51	42
	Elliott's B	34	0.124	0.000	†	—	3.8	98.4	—	168	—	30	—
	Methotrexate, experiment 2	8	0.125	0.119	199	7.5	3.7	100.9	102.0	138	192	24	42
	Elliott's B	15	0.123	0.112	†	—	3.7	99.9	—	212	—	36	—
	Elliott's B	10	0.128	0.082	179	—	3.8	101.2	98.6	240	208	39	57

*Methotrexate Ci concentration, 0.3 mg/ml. (For details of perfusion procedures *see* Materials and Methods.)
†Perfusions discontinued for technical reasons.

TABLE 2.—Perfusion characteristics of monkeys during VL subarachnoid space perfusions with methotrexate*

Monkey No./sex	Perfusion	Average opening ventricular pressure (cm H_2O)	Range of perfusion pressures (cm H_2O)	^{3}H-inulin equivalents clearance (μl/min)	CSF rate of formation (μl/min)
156-KK/M	Elliott's B	3.0	3–12	68	27
	Elliott's B	(<0)	4–12	—	—
	Methotrexate, experiment 1	6.5	4–12	—	—
	Elliott's B	5.0	7–26	61	75
	Methotrexate, experiment 2	13.0	29–46	—	—
	Elliott's B	9.0	12–28	64	64
157-KK/M	Elliott's B	6.0	6–20	87	49
	Elliott's B	17.5	6–14	70	55
	Methotrexate, experiment 1	0	7–14	—	—
	Elliott's B	9.5	13–47	—	—
	Methotrexate, experiment 2	2.5	2–15	—	—
	Elliott's B	-2.5	7–30	—	—
	Elliott's B	(<0)	5–24	86	81

*Methotrexate Ci concentration, 0.3 mg/ml. Intervals between perfusions, Ci rates, Co rates, durations of perfusions, and amounts of drug perfused are listed in table 1.

During and after Elliott's B perfusions the neurologic status of both monkeys was judged to be normal, ie, animals did not convulse, display tremors, or develop motor deficits.

TABLE 3.—Lumbar Co concentration vs left ventricular Ci concentration and recovery of methotrexate from lumbar Co samples during perfusion of methotrexate through VL space of monkeys

Monkey No./sex	Perfusion	^{3}H-inulin equivalents (Co/Ci)	Methotrexate	
			Co/Ci	% recovery from CSF
156-KK/M	Elliott's B	0.80	—	—
	Elliott's B	0.69	—	—
	Methotrexate, experiment 1	—	0.92	83.7
	Elliott's B	0.58	—	—
	Methotrexate, experiment 2	—	0.52	30.0
	Elliott's B	0.61	—	—
157-KK/M	Elliott's B	0.66	—	—
	Elliott's B	0.65	—	—
	Methotrexate, experiment 1	—	0.43	45.9
	Elliott's B	—	—	—
	Methotrexate, experiment 2	—	0.70	70.2
	Elliott's B	—	—	—
	Elliott's B	0.71	—	—

*Methotrexate Ci concentration, 0.3 mg/ml. Intervals between perfusion, Ci rates, Co rates, durations of perfusions, and amounts of drug are listed in table 1.

Methotrexate Perfusions

Vital signs.—During methotrexate perfusion experiments, heart rates, respiratory rates, and body temperatures at the end of each perfusion were judged to be within the limits associated with non-drug Elliott's B perfusion (table 1).

Perfusion characteristics.—With the exception of one perfusion dose in monkey 156-KK, perfusion pressures and lumbar Co rates during drug perfusions were within the range associated with Elliott's B perfusions (table 2).

Methotrexate concentration in lumbar Co samples during perfusion.—The concentration of methotrexate in lumbar Co samples for three of four experiments reached a plateau in about 60 minutes (figs 1A, 1B, and 2A). During the plateau period for these three experiments, methotrexate concentration (Co) averaged about 5.0×10^{-4} M, and the Co/Ci ratios ranged from 0.92 to 0.43 (table 3). In the one drug perfusion experiment in which a distinctive plateau was not obtained (fig 1B), the average Co/Ci value for the final 60 minutes of the perfusion was 0.52 (table 3), and in this experiment perfusion pressures were abnormally high (table 2).

Percentage methotrexate recovery from lumbar Co.—The average percentage recovery of methotrexate ranged from 30.0% to 83.7% (table 3). The smallest drug recovery value of 30.0%, which was obtained for monkey 156-KK, was associated with relatively high perfusion pressures (29–46 cm H_2O)

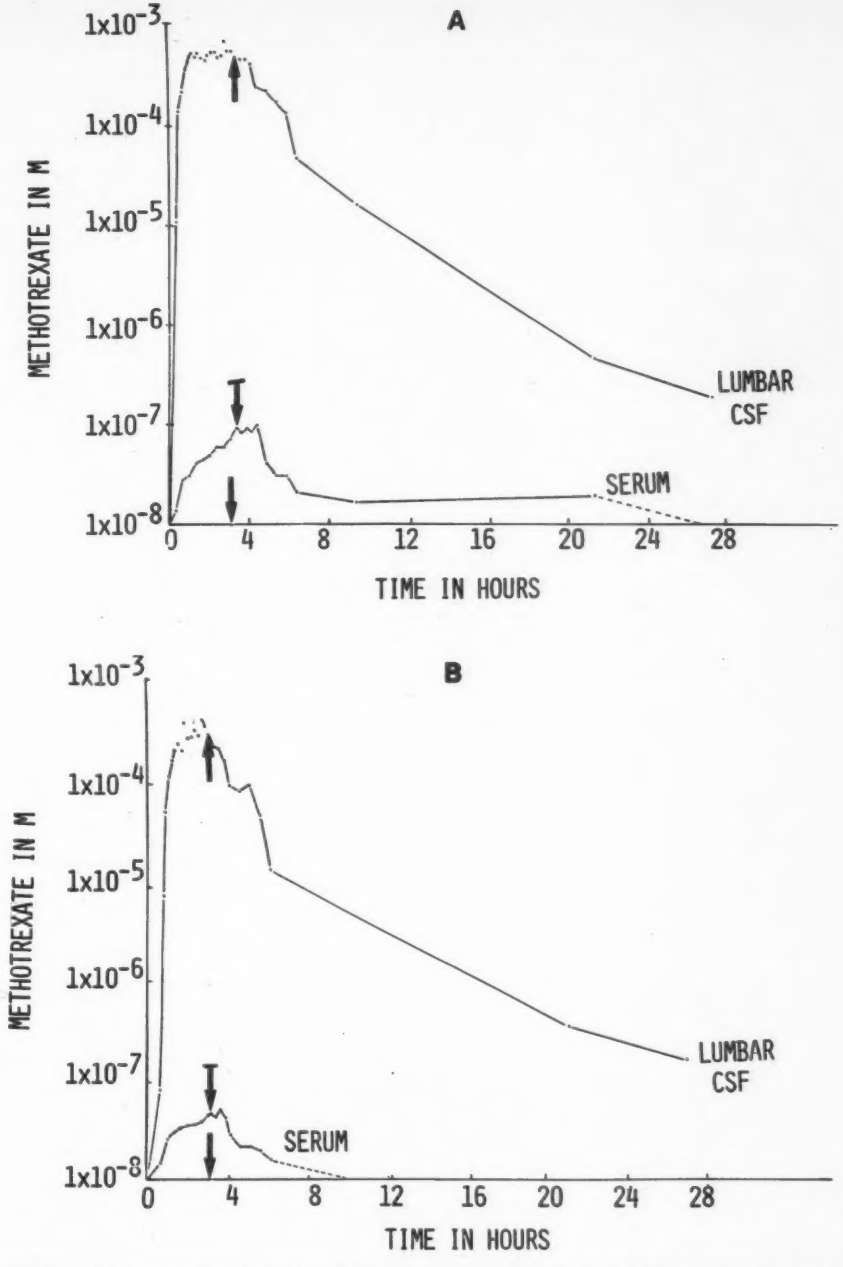

FIGURE 1.—Methotrexate concentration in lumbar CSF and plasma samples during and following left VL subarachnoid space perfusions of methotrexate in monkey 156-KK. ↓ = end of perfusion period; beginning of post-perfusion sampling; ↑ = lumbar CSF; T = serum. A = experiment 1; B = experiment 2.

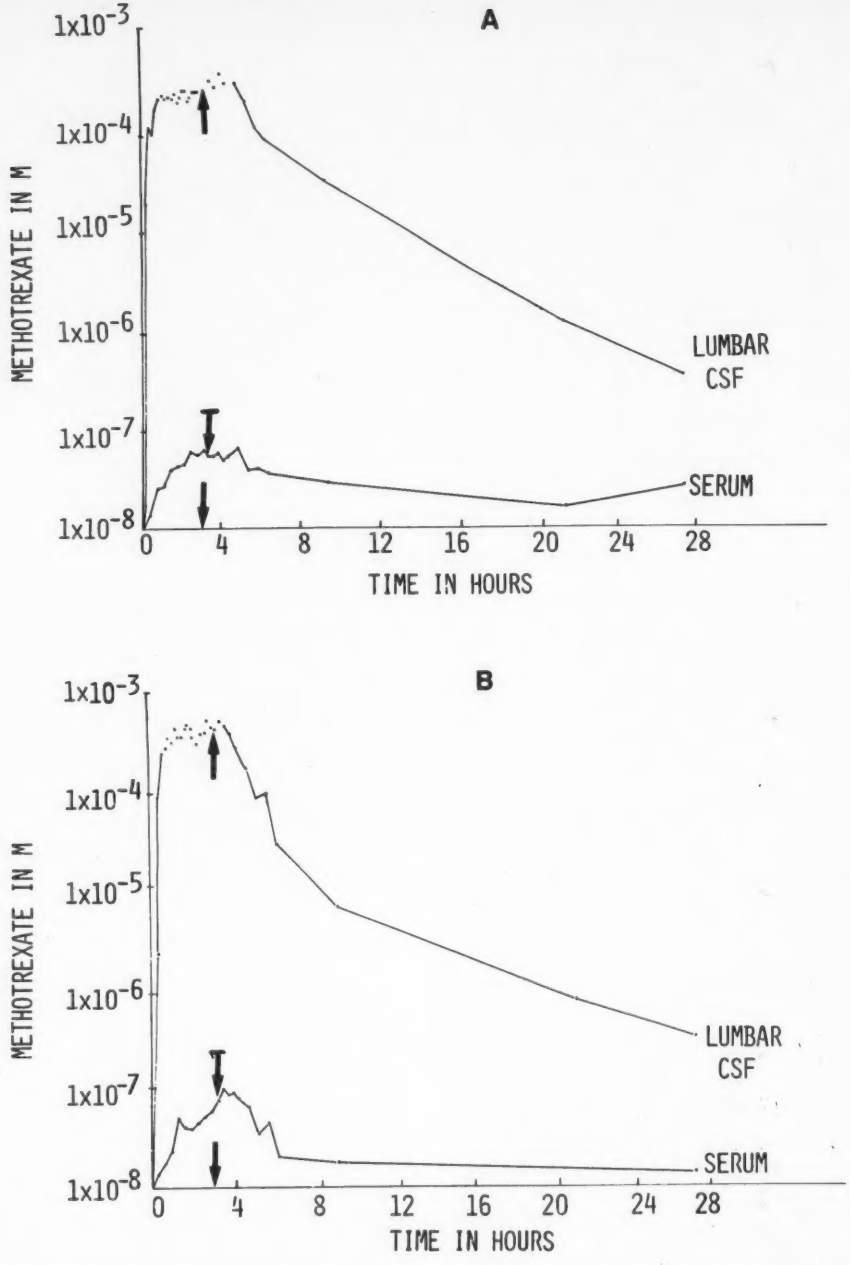

FIGURE 2.—Methotrexate concentration in lumbar CSF and plasma samples during the following left VL subarachnoid space perfusions of methotrexate in monkey 157-KK. ↓ = end of perfusion period; beginning of post-perfusion sampling; ↑ = lumbar CSF; ↧ = serum. A = experiment 1; B = experiment 2.

TABLE 4.—Elimination kinetics of methotrexate in CSF of monkeys following perfusion of the left VL subarachnoid space*

	Lumbar CSF			
	Methotrexate concentration		Half-life (mins)	
Monkey No.	Initial ($\times 10^{-4}$ M)	24 hrs ($\times 10^{-7}$ M)	1st phase	2nd phase
156-KK				
Experiment 1	5.2	2.0	45	135
Experiment 2	2.5	1.6	45	195
157-KK				
Experiment 1	2.5	3.5	45	135
Experiment 2	4.0	3.3	40	210

*See figs 1A, 1B, 2A, and 2B for CSF-methotrexate concentration × time curves from which data were obtained. Lumbar CSF samples were used for the analysis of methotrexate. (For details of perfusion and analytic procedures *see* Materials and Methods.)

TABLE 5.—Elimination kinetics of methotrexate in plasma of monkeys perfused through the left VL subarachnoid space

	Plasma			
	Methotrexate concentration		Half-life (mins)	
Monkey No.	Initial ($\times 10^{-8}$ M)	24-hrs ($\times 10^{-8}$ M)	1st phase	2nd phase
156-KK				
Experiment 1	9.5	<1.0	60	960
Experiment 2	4.5	<1.0	45	—
157-KK				
Experiment 1	5.5	2.5	40	630
Experiment 2	8.0	1.4	75	—

*See figs 1A, 1B, 2A, and 2B for plasma-methotrexate concentration × time curves from which data were obtained. (For details of perfusion and analytic procedures *see* Materials and Methods.)

and a relatively low lumbar Co rate (0.082 ml/minute). Data otherwise show that percentage recoveries, within the range of 45.9%–83.7%, were associated with Co rates of 0.119–0.167 ml/minute and perfusion pressures of 2–15 cm H_2O.

Kinetics of elimination of methotrexate from CSF space following VL drug perfusion.—The concentration of methotrexate in lumbar Co at the start of the post-perfusion collection period ranged from 2.5 to 5.2×10^{-4} M (table 4). The elimination of methotrexate from the CSF space of monkeys occurred in two phases (figs 1A, 1B, 2A, and 2B): the initial or rapid phase took place during the first 3 hours; during this period about 90% of the drug disappeared and the half-life was estimated to be about 45 minutes. The second or slow phase of elimination occurred between the 3rd and 24th hours following the perfusion; during this period about 99% of the remaining drug disappeared and the half-life during this phase was estimated to be about 3 hours. At 24 hours, lumbar samples contained an average of about 2.5×10^{-7} M methotrexate (table 4).

Methotrexate concentration in plasma.—The plasma concentration of methotrexate reached a plateau in about 140–180 minutes after the start of VL perfusion, and the average concentration of drug during the plasma plateau period was approximately 5.0×10^{-8} M (figs 1A, 1B, 2A, and 2B). At the start of the post-perfusion period, plasma levels of methotrexate ranged from 4.5 to 9.5×10^{-8} M (table 5) or about 0.02% of lumbar CSF concentration. The disappearance of methotrexate from plasma following perfusion tended to occur in two phases (figs 1A, 1B, 2A, and 2B): the rapid phase took place during the initial 2–3-hour post-perfusion period; during this period the half-life was estimated to be about 60 minutes. Following this period, the concentration of drug in plasma tended to decline slowly with the half-life estimated to be as long as 16 hours in one experiment (table 5).

Delayed effects of methotrexate perfusions.—At intervals varying from 15 to 34 days following methotrexate perfusion, the VL space was perfused with Elliott's B solution containing trace amounts of radiolabeled inulin. For monkey 156-KK, data showed that perfusion characteristics, CSF rate of formation, and inulin clearance were not adversely affected by previous methotrexate perfusions (table 2). For monkey 157-KK, perfusion characteristics were found to be within the pre-drug range 25 days following the second drug perfusion, although values tended to be somewhat outside normal limits at intervening time intervals (table 2). The neurologic status of the two monkeys was judged to be within acceptable limits during post-perfusion periods, ie, animals did not develop convulsions, tremors, or motor deficits.

DISCUSSION

The pharmacokinetics of methotrexate in the CSF of monkeys were studied following VL perfusion. The perfusion procedure was used rather than a single injection of drug given intraventricularly or intrathecally primarily because the drug could be administered until the ratio of Co to Ci methotrexate concentrations had reached a plateau value. The pharmacokinetic study of methotrexate during the post-perfusion period, therefore, began when drug concentration was in apparent "equilibrium" within the perfused VL perfusion route.

The methotrexate Co/Ci values, which attained a

plateau in about 60 minutes, were quite similar to those previously reported for the monkey (12) using trace amounts of radiolabeled methotrexate as a marker, but they were higher than the reported Co/Ci value obtained by Rubin et al (13) during perfusion of the ventriculocisternal (VC) space of the dog. The higher Co/Ci values recorded in the current studies with VL perfused monkeys may be due to the higher Ci concentration of methotrexate used, although species differences, rates, and routes of perfusion cannot be ruled out as alternate reasons.

The apparent similarity between the Co/Ci values of methotrexate and radiolabeled inulin found in these experiments has been noted previously for ^{3}H-methotrexate and radiolabeled inulin (12). This suggests that a major pathway for the absorption of methotrexate from the perfused VL space of the monkey occurred via the same bulk-flow pathways by which inulin is absorbed (8), although alternate pathways are probably involved, such as active transport of methotrexate by the choroid plexus (13).

The average percentage recovery of methotrexate was 57% ± 14%. In other studies done in our laboratories which used radiolabeled ^{3}H-methotrexate for analytic purposes, the average percentage recovery of ^{3}H-methotrexate equivalents for VL perfusions was 55% ± 10% for Ci concentrations ranging from 1.2 to 0.15 mg/ml. Accordingly, present results obtained using a microbiologic assay are comparable to those obtained using radiolabeled methotrexate.

The elimination of methotrexate from the perfused VL space of the monkey occurred in two phases: a rapid first phase with a half-life of 45 minutes and a relatively slow second phase with a half-life of about 170 minutes. Data for the elimination of ^{3}H-inulin from the perfused VL space of the monkey which were obtained from other experiments not described in this paper have also shown two phases with respective half-lives of 75 ± 23 minutes and 12 ± 5 hours. The occurrence of a relatively fast phase of elimination following VL perfusion, therefore, may be a common phenomenon, especially for those drugs that have Co/Ci values similar to inulin during perfusion. A fast phase would be reasonably consistent with a prediction made from a one-compartment flow-through model for which the rate of removal of a substance is proportional to the ratio of the rate of outflow to the volume of the compartment, and in which the units of volume/time are considered to be the clearance rate of a substance introduced into the compartment. In the case of inulin, the predicted half-life would be approximately 40 minutes assuming a VL space of 4 ml and a clearance of 0.070 ml/minute.

The persistence of methotrexate in the CSF space of the monkey during these experiments may be partially attributable to feedback from the plasma pool, as suggested by the data of Rubin et al (13) for the dog and data of Tattersall et al (14) for man. However, the average plasma concentration of drug in the monkey was, at most, 0.02% of the average amounts contained within the CSF space immediately following the perfusion. Thus, feedback of methotrexate from blood to CSF was not a major factor in maintaining drug levels within the CSF space. It is probable that the prolonged CSF levels found in these studies may be more appropriately related to the pool of methotrexate contained within the periventricular tissues of the brain following perfusion of the ventricular space with drug rather than feedback from the blood compartment (15).

Bleyer et al (1) have indicated that the actual concentration of methotrexate in the CSF of patients who show signs of toxicity was quite low (10^{-8} to 10^{-7} M) 2 days after intrathecal administration of drug. In our monkey experiments, CSF concentrations attained during the plateau phase of perfusion were, on an average, 3.0×10^{-4} M, but animals did not show signs of neurotoxicity. This indicates a difference in sensitivity of the monkey central nervous system to acute methotrexate toxicity, although the monkey does show evidence of neurotoxicity when the VC and VL spaces are perfused with Ci concentrations of methotrexate $\geq$ 0.6 mg/ml (1.32×10^{-3} M) (16). However, the importance of prolonged exposure to low concentrations of methotrexate cannot be ignored as discussed by Bleyer et al (1).

The rather prolonged plasma half-life (10.5–16 hours) found during the slow phase of methotrexate elimination following VL perfusion has not been reported to occur in the monkey when ^{3}H-methotrexate is given systemically (17). However, in the study that used ^{3}H-methotrexate, data for terminal half-life are difficult to interpret because of the extensive accumulation of metabolites. Data from our current experiments suggest that methotrexate in the CSF space may act as a reservoir from which plasma levels of methotrexate were maintained. This would be in accord with the findings of Rubin et al (13) for methotrexate and Rall et al (18) for aminopterin given intrathecally to the dog.

The current studies have not described the neuropharmacologic responses associated with methotrexate perfusions in monkeys. Detailed studies, however, have been done in monkeys perfused through the VC and VL routes (12,16,19,20). It should be noted that monkeys perfused with methotrexate do show gross neurologic toxicity, principally in the form of seizures and hypokinesia with occasional residual motor deficits (20). These obser-

vations are somewhat similar to neurotoxicity reported to occur in the dog (18) and man (1). In addition, certain monkeys perfused through the VL and VC routes with methotrexate have been found to have a severe necrotizing leukoencephalopathy (20). In this regard the occurrences of brain lesions in man associated with intraventricular instillation of methotrexate have been reported by Shapiro et al (21) and Bresnan et al (22).

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Perilymph Penetration of Methotrexate in Cats [1,2]

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SUMMARY

The perilymph of the inner ear has recently been suggested as a site of frequent leukemic or lymphomatous infiltrates. Experimental evidence supports the existence of barrier mechanisms and explains the attenuated entry of several substances, including chemotherapeutic agents, from either the cerebrospinal fluid or blood into the perilymph. The present study describes the attenuated entry of methotrexate into the perilymph compartment of experimental animals following intrathecal or iv administration.

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At the time of death, approximately 60%–70% of patients with childhood leukemia have evidence of intracranial involvement (1). An area of significant intracranial involvement which has only recently been explored is the temporal bone. Histopathologic examination of temporal bones removed at autopsy from patients dying of various forms of leukemia has disclosed leukemic cells in the inner ear fluids. To date, the literature contains reports of 47 patients with leukemia whose temporal bones had been removed for histopathologic study (2–9). Six patients (13%) had leukemic cells, often in large numbers, in the endolymph or perilymph of the inner ear. Similar reports of seven patients with non-Hodgkin's lymphomas (10,11) reveal that three patients (43%) showed lymphomatous cells in the inner ear fluids.

Aside from the cochlear or vestibular symptoms which such malignant invasions can cause, the presence of leukemic or lymphomatous cells in the inner ear fluids implies that the inner ear fluids may qualify as a pharmacologic sanctuary. Experimental evidence indicates that the ionic composition, protein and enzyme content, osmotic pressure, and pH of the perilymph are different from those of either serum or cerebrospinal fluid (CSF) (12–14). Further, there are only minor exchanges of ions between the CSF and the perilymph (15) even though an anatomic communication, the cochlear aqueduct, exists between these two fluid compartments. Thus, the existing evidence strongly argues in favor of the concepts of both a blood-perilymph barrier and a CSF-perilymph barrier.

The purpose of the present study was to evaluate the fate of intrathecally and iv injected methotrexate (MTX) in the perilymph of the inner ear, in order to establish the relationship of MTX concentration in the perilymph relative to its concentration either in CSF or serum.

MATERIALS AND METHODS

Mongrel cats were anesthetized with sodium pentobarbital (30 mg/kg) and received either an intrathecal injection of MTX through the atlantooccipital membrane at a dose of 15 mg/m^2 or an iv "push" infusion of MTX at a dose of 1 g/m^2. All animals receiving MTX intrathecally were killed 4 hours later and samples of serum, CSF, and perilymph were removed for determination of MTX concentrations. Groups of animals receiving MTX iv were killed 4, 8, 12, 24, 48, 72, and 96 hours after injection, and identical samples were obtained.

A modified microbiologic assay (16) was used for the determination of MTX concentrations in the

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[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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body fluids. Using a disc-assay technique similar to that designed for antibiotic assays, a standard curve of MTX concentration is prepared by using purified MTX in solutions of 5, 10, 30, and 50 ng/ml which have been applied to filter paper discs (6.5 mm in diameter) in 0.02-ml quantities. The unknown samples are similarly applied to filter paper discs following necessary dilutions and allowed to dry for 4 hours at 37°C. The assay plates containing 10 ml of Flynn medium (17), 0.5 ng/ml of folic acid, and 1.5% agar are pre-seeded with *Streptococcus faecium* var. *durans*. The dried discs containing standard MTX concentrations and the unknown samples are then placed on the solidified agar which had been precooled at 4°C for ≥ 2–4 hours. The plates are then incubated for 18 hours at 37°C and zones of growth inhibition are observed on the plates around the filter paper discs; the size of the zone is proportional to the concentration of MTX. A standard curve is derived by measuring the diameter of the zone of inhibition and plotting it against the logarithm of known concentrations of the drug. The concentrations of the unknown samples tested routinely in triplicate are then quantitated from the standard curve.

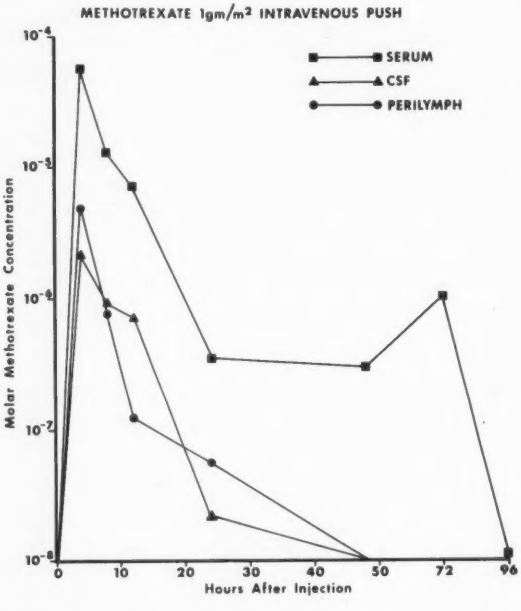

FIGURE 1.—Mean molar MTX concentration, on a logarithmic scale, of serum, CSF, and perilymph at various times after an iv push of 1 g/m² of the drug. Each point represents the mean of 3 or 4 animals.

RESULTS

Nine animals received intrathecal MTX at a dose of 15 mg/m². Sampling body fluids 4 hours later showed that the mean MTX concentration of the CSF was 2.21×10^{-4} M, while the mean MTX concentration of the perilymph was significantly lower at 2.37×10^{-5} M. In addition, four of the nine animals showed perilymph MTX concentrations $< 6.0 \times 10^{-6}$ M, and two of these showed perilymph concentrations $< 1.20 \times 10^{-7}$ M. The lower concentrations of MTX are probably the most meaningful in this study, for it is technically quite difficult to obtain samples of perilymph which are not contaminated with CSF. As perilymph is aspirated from the round window of the animal, there is a great tendency to aspirate CSF which can be drawn through the cochlear aqueduct into the perilymph compartment.

After iv doses of MTX of 1 g/m², both CSF- and perilymph-MTX concentrations tend to be similar at all times of sampling (fig 1), but are consistently lower than the simultaneous serum concentrations. MTX enters the perilymph compartment with this method of administration, but the highest mean concentration in the perilymph achieved, 4.88×10^{-6} M 4 hours after injection, was one tenth the simultaneous serum concentration.

DISCUSSION

The data generated by this study support the concept of a CSF-perilymph barrier for MTX since 4 hours after an intrathecal injection of the drug MTX is present in the perilymph at concentrations of about ten to 100 times less than those simultaneously observed in the CSF. Our findings with a small number of animals that received intrathecal cytosine arabinoside at a dose of 50 mg/m² parallel the MTX data. Employing a similar microbiologic assay (18), cytosine arabinoside was detected in the perilymph 4 hours after injection at significantly lower concentrations than those simultaneously observed in the CSF.

The fate of intrathecally administered MTX is similar to that of other substances studied. The total protein content of the perilymph in experimental animals increases when the protein content of the CSF is increased by an intracisternal injection of albumin; however, the protein concentration never exceeds two thirds that of the CSF. A similar pattern is also evident for intracisternally administered urea with the maximum perilymph-urea concentration approaching only 50% that of the CSF (19).

Iv administered high-dose MTX also entered the perilymph compartment poorly; the mean perilymph-MTX concentrations consistently remained approximately ten times less than those simultaneously observed in the serum. Similarly, when a solution of 40% glucose is injected iv into experimental animals, the maximum glucose concentration achieved in the perilymph is only about one third of the maximum achieved in the serum (20).

Our findings strongly support the idea that, as with other substances so studied, both a CSF-perilymph barrier and a blood-perilymph barrier exist for MTX. As a consequence, the possibility that the perilymph compartment may qualify as a pharmacologic sanctuary for leukemic or lymphomatous cells becomes more real depending, of course, on the sensitivities to MTX of the malignant cells which are sequestered in this compartment (21–24). Because of the apparent CSF-perilymph and blood-perilymph barriers, target cells in this compartment may not be able to accumulate sufficient levels of free intracellular MTX (25), thus precluding in certain cases the cytotoxic action of this drug.

Extrapolating the findings of this animal system to the situation in man, the question must be entertained whether patients who have therapy for the treatment or prophylaxis of central nervous system leukemia will experience maximum benefit from regimens which depend on the penetration of intrathecal or high-dose iv MTX into the perilymph compartment. Malignant cells sequestered in this compartment may not be optimally exposed to the cytotoxic action of this drug. It is possible that therapeutic regimens which include cranial irradiation (the effects of which are not at all influenced by these barrier mechanisms) may be more efficacious in this regard. In addition, malignant cells in this compartment which are being exposed to sublethal levels of chemotherapeutic agents might well be the cell population from which drug-resistant cell lines could likely emerge.

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Comparison of Tissue Distribution and Fate of 2,2′-Anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine and 1-β-D-Arabinofuranosyl-5-fluorocytosine in Rats[1]

Ting-Chao Chou,* Pedro Vidal, and Frederick S. Philips[2]

SUMMARY

The distribution and fate of (^{14}C-2)-2,2′-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine (AAFC) and its hydrolyzed product, (^{14}C-2)-1-β-D-arabinofuranosyl-5-fluorocytosine (AFC) were compared in rats. Recovery of radioactivity in urine 0–24 hours after single iv injections of AAFC was 85%, of which 78% was unchanged drug and 14% was AFC; recovery of AFC was 90%, of which 89% was unchanged AFC and 7% was 1-β-D-arabinofuranosyl-5-fluorouracil. The concentration of the drugs was determined in tissues at various times after iv injection and was expressed in terms of distribution factors (DF) assuming the initial dose per g of body weight to be unity. Log DF versus time followed first-order elimination kinetics in most tissues of rats given AFC with plasma half-life being 50 minutes, whereas AAFC was eliminated rapidly during initial phases (half-life, 15 minutes) followed by much slower rates. The initial volume of distribution for AAFC was considerably smaller than for AFC. The DF value in the brain after AAFC was 0.03, was maximal at 15 minutes, and decreased by half within 30 minutes; after AFC, the DF in the brain was 0.075 at 15 minutes, increased steadily to 0.15 at 90 minutes, and thereafter decreased slowly. The distributions after AFC were identical in plasma, skeletal muscle, and heart from 0 to 4 hours, whereas at 4 hours after AAFC, the DF in the heart was 15-fold higher than in plasma and 30-fold higher than in skeletal muscle. These data indicate that AAFC and AFC exhibit substantially different distribution patterns and that the protonated AAFC, in contrast to AFC, does not readily equilibrate with total body water. AAFC showed acute toxicity in rats which was not observed for AFC. Complete hydrolysis of AAFC indicated that 13% could not be accounted for as AFC, but appeared as breakdown products of the pyrimidine ring. One degradation product was subsequently identified by Otter and Fox as fluoromalonaldehydic acid which would be oxidized in vivo to fluoromalonic acid and ultimately to fluoroacetate, compounds known to inhibit the Krebs cycle. As expected, injections of AAFC and of low doses of hydrolyzed AAFC drastically increased citrate levels in rat tissues, whereas AFC was devoid of this effect.

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The usefulness of cytosine arabinoside (ara-C) in treating leukemia in both experimental animal systems and in man is well known. The 5-fluoro-analog of ara-C, 1-β-D-arabinofuranosyl-5-fluorocytosine (AFC), has also been shown active against mouse leukemia (1,2). Both compounds probably share the same mode of action since their cytotoxic effect can be reversed by deoxycytidine (1–4). Both also serve as substrate for pyrimidine nucleoside deaminase (2). The resultant 1-β-D-arabinofuranosyl-5-fluorouracil (AFU), unlike arabinoside uracil (ara-U), is chemotherapeutically active with its effect similar

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to that of 5-fluorouracil (5-FU) (3). 2,2'-Anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine (AAFC) and 2,2'-anhydro-1-β-D-arabinofuranosylcytosine (anhydro-ara-C) are both effective antineoplastic agents in experimental animals (5–7) and in man (7,8). AAFC and anhydro-ara-C are resistant to enzymatic deamination and can undergo slow non-enzymatic hydrolysis to produce AFC and ara-C respectively, thus serving as depots of chemotherapeutically active precursors (9,10).

The ionic properties of AAFC and AFC at physiologic pH, as shown in figure 1, are quite different. Since AAFC is a strong base, it is a cation at physiologic pH and would, thus, be less permeable to cell membranes on the blood-brain barrier than AFC. In this paper, we show that the tissue distribution and fate of AAFC and AFC in rats are, in fact, different. Rats were used for the studies since, in contrast to other animal species, their tissues have negligible pyrimidine nucleoside deaminase activity (11).

MATERIALS AND METHODS

AFC (1) and AAFC hydrochloride (3) were synthesized by Dr. Jack J. Fox of the Sloan-Kettering Institute. ^{14}C-2-AAFC monohydrochloride (9.4 microcuries/mg) was kindly provided by Dr. A. A. Liebman of Hoffman-La Roche, Inc, Nutley, NJ. It was tested by cellulose thin-layer chromatography using two solvent systems: (*a*) *n*-propanol:ethylacetate, 1:2, saturated with 0.05 M acetic acid, and (*b*) isopropanol:0.1 M HCl, 7:3, in which the R_f values were 0.1 and 0.55 respectively. Radioactivity recovered in the AAFC zone using system (*a*) was 99.4% and in system (*b*) was 98.4%.

^{14}C-2-AFC was prepared by neutralizing aqueous solutions of ^{14}C-2-AAFC hydrochloride with dilute NaOH to pH 8 and incubating at 50°C for 1 hour.

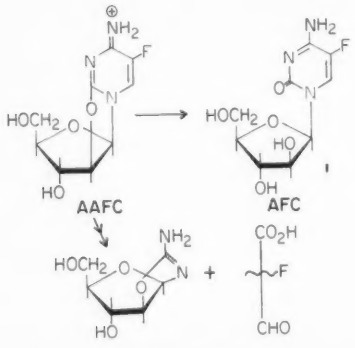

FIGURE 1.—Structure of AAFC and its hydrolytic products.

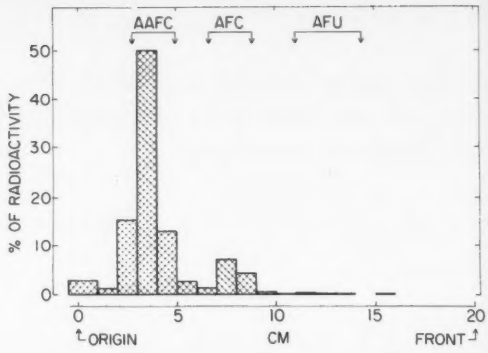

FIGURE 2.—Typical paper chromatogram of 0–24-hr urine collected from a rat receiving an iv injection of ^{14}C-2-AAFC (2 mg, 10 microcuries/kg). Urine was collected as described in Materials and Methods. Solvent system (*a*), *n*-propanol:ethylacetate, 1:2, saturated with 0.05 M acetic acid, was used for descending chromatography using Whatman No. 3 paper. The zones for non-labeled reference compounds, as shown for AAFC, AFC, and AFU, were detected as ultraviolet-absorbing spots.

The hydrolyzed preparation was streaked on Whatman No. 3 paper and chromatographed (descending) in solvent system (*a*). The AFC zone, R_f 0.34–0.40, was eluted with H_2O; the eluate was concentrated by flash evaporation. The ultraviolet spectrum was identical with that of authentic AFC and in cellulose thin-layer chromatograms, developed with solvent system (*a*), 94.7% of radioactivity was recovered in the AFC zone. The specific activity was found to be 10.2 microcuries/mg.

Tissues and feces were homogenized with 10% cold perchloric acid and the radioactivity in acid-soluble extracts was measured. Radioactivity was determined as previously described (12) using a Packard Tri-Carb model 3375 liquid scintillation spectrophotometer. Counting efficiencies were automatically obtained for samples and used for calculation of dpm. Drug concentrations, in μg/ml or μg/g of wet tissues, were calculated from the values for radioactivity and were expressed in terms of distribution factors (DF) assuming the initial dose per g of body weight to be unity (13).

The radioactivity in nucleoside and in nucleotide fraction in tissues at various times after an iv injection of ^{14}C-2-AAFC was measured using an anionic exchange column procedure previously used for study of the same fractions in tissues of animals given ara-C (12). Similar to ara-C, AAFC as well as AFC appeared in the void volume of the columns. All anionic substances, presumed to be phosphorylated nucleosides because they were adsorbed by the resin, were eluted with 1 M ammonium bicarbonate (12).

The citrate levels in tissues were measured by the method of Beutler and Yeh (14).

Male CD rats were purchased from the Charles River Breeding Laboratories, Wilmington, Mass; they were used when they were 7–8 weeks old. When urine was to be collected, each rat was placed in a metabolic cage where it had free access to food and water. The urine receivers were iced and each contained 1 ml of 1 M acetic acid to suppress spontaneous hydrolysis of AAFC. The urine collections were diluted to contain 0.05 M acetic acid before sampling for measurement of radioactivity and for chromatography. Solvent system (*a*) was used to develop paper chromatograms of urine samples obtained from animals receiving either ^{14}C-2-AAFC or ^{14}C-2-AFC. As shown in figure 2, the R_f values for authentic AAFC, AFC, and AFU were 0.20, 0.38, and 0.64 respectively.

RESULTS AND DISCUSSION

Excretion of AAFC and AFC

Within the first 24 hours, about 85% of radioactivity appeared in urine from rats given ^{14}C-2-AAFC and about 90% from animals receiving ^{14}C-2-AFC (table 1). Recovery in urine was negligible during the second 24-hour period. Radioactivity in feces between 0 and 48 hours amounted to only 2%–4% of the dose. The percentage recovery of AAFC in both urine and feces was independent of dose over the 100-fold range examined.

Each of the urines collected from the rats in table 1 between 0 and 24 hours was chromatographed (fig 2). For the nine rats receiving different doses of ^{14}C-2-AAFC, 77.6% ± 6.1% of radioactivity appeared in the AAFC zone, 13.6% ± 3.1% in the AFC zone, and 0.6% ± 0.2% in the AFU zone. A total of 91.7% ± 5.4% of the radioactivity was accounted for in these zones. For the urines from the three rats given ^{14}C-2-AFC, 88.6% ± 1.0% was found in the AFC zone and 6.9% ± 1.1% in the AFU zone. A total of 95.5% of radioactivity was accounted for in these two zones.

Distribution of AAFC and AFC in Body Fluids and Tissues

The concentrations of AAFC and AFC in tissues were determined and compared at various times after iv injection and the data were expressed in terms of DF (figs 3 and 4). For rats given ^{14}C-2-AFC, plots of log DF versus time showed first-order elimination kinetics in plasma, muscle, heart, kidney, liver, spleen, and lung with similar half-lives (as determined by linear regression) that varied from a minimum of 49 minutes in the plasma to a maximum of 61 minutes in the spleen. From the similarity in the slopes of these plots and from their linearity, it may be concluded that AFC rapidly diffuses into and out of the tissues of the above organs.

The virtual volume of distribution of AFC, obtained by extrapolating plasma values to 0 time, was 790 ml/kg; this value suggests that the substance is rapidly equilibrated in body water. The rapid diffusion of AFC into cells was further shown by the fact that the initial DF values for all organs except kidney and brain were close to unity. The higher initial values for kidney were probably due to concentration of AFC in tubular urine. The low initial value in the brain shows that AFC, though highly diffusible into most tissues, moves across the blood-brain barrier relatively slowly. With regard to movement across this barrier, it is also interesting to note that efflux from the brain was also relatively slow (compare DF values in the brain and plasma at 3 and 4 hours). Possible factors responsible for the apparent retention of AFC by intestine and thymus (fig 4) will be discussed later.

Quite different results were obtained in rats given ^{14}C-2-AAFC. There were no organs except the heart in which plots of log DF versus time were linear (figs 3 and 4). The initial rates of elimination from the plasma, muscle, kidney, and liver were considerably greater than those found with AFC. For example, estimation of half-lives between 10 and 30 minutes after injection were < 20 minutes in each of these organs (fig 3). At later times, the rates of

TABLE 1.—Recovery of radioactivity in urine and feces of rats receiving iv injections of ^{14}C-2-AAFC or ^{14}C-2-AFC*

Compound	Dose (mg/kg)	Urine		Feces	
		0–24 hrs	24–48 hrs	0–24 hrs	24–48 hrs
AAFC	200	86.0 ± 6.7	0.2 ± 0.03	3.4 ± 3.5	0.6 ± 0.4
	20	81.3 ± 11.3	0.3 ± 0.07	2.9 ± 1.6	0.9 ± 0.8
	2	86.1 ± 8.1	0.3 ± 0.03	3.1 ± 1.7	0.8 ± 0.9
AFC	20	90.1 ± 2.5	0.2 ± 0.06	2.4 ± 3.6	0

*The data are given in % of dose. The values are means ± 1 SD for groups of 3 rats. *See* Materials and Methods for details.

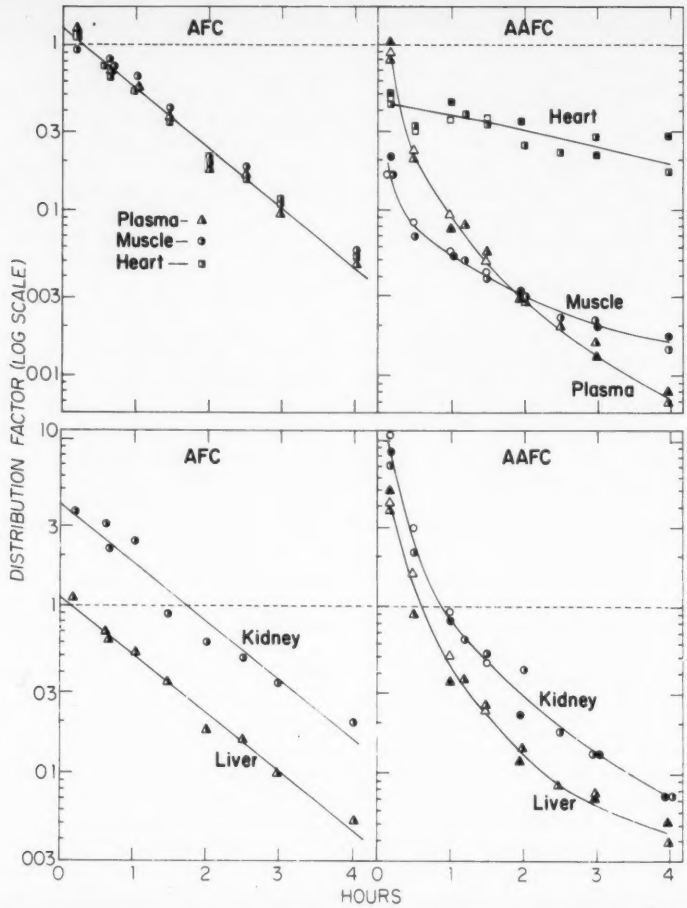

FIGURE 3.—Distribution of acid-soluble radioactivity in the plasma, skeletal muscle, heart, kidney, and liver of rats given iv injections of ^{14}C-2-AAFC and ^{14}C-2-AFC. The dose of ^{14}C-2-AFC for all animals was 20 mg/kg containing 4.8 microcuries/kg of radioactivity. The doses of ^{14}C-2-AAFC were: open symbols, 2 mg/kg; half-filled symbols, 20 mg/kg; and filled symbols, 200 mg/kg. Each dose contained 10 microcuries/kg of radioactivity. Drug concentrations, μg/g of wet tissue or μg/ml of plasma, were calculated from the values for radioactivity and were expressed in terms of DF assuming the initial dose per g of body weight to be unity (ie, the horizontal broken line). Each symbol represents the value obtained from an organ from 1 rat.

elimination from plasma, muscle, kidney, and liver became perceptibly slower. Recent studies in which ^{14}C-2-AAFC was injected into leukemic patients also showed nonlinear elimination of radioactivity from plasma (15).

The initial uptake values differed widely among the organs. The earliest DF values in kidney and liver were much greater than unity and also greater than the values obtained after giving rats ^{14}C-2-AFC. The DF curves for the plasma, kidney, and liver were parallel throughout the 4-hour period when concentrations were falling 100-fold.

The fact that the initial DF values for kidney in animals receiving AAFC were considerably greater than in those given AFC (fig 3) suggests that the rate of renal excretion of the former is greater than that of the latter. Similar comparisons of initial DF values in the liver suggested the possibility that AAFC was concentrated in bile. However, in two rats in which the common duct was cannulated with the animals under pentobarbital anesthesia, the DF values in bile during the first hour after iv injection of ^{14}C-2-AAFC were 0.21 and 0.50 and, in the second hour, 0.08 and 0.13. These low values do not provide evidence for biliary concentration.

The initial uptake values in the muscle, spleen,

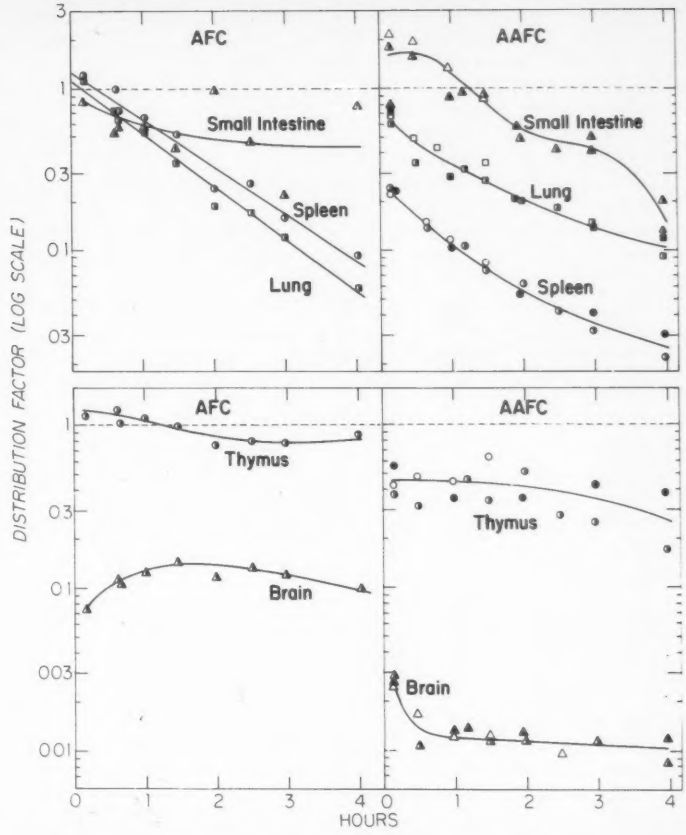

FIGURE 4.—Distribution of acid-soluble radioactivity in the small intestine, spleen, lung, thymus, and brain of rats given iv injections of ^{14}C-2-AAFC and ^{14}C-2-AFC. The dose(s) and the symbols for each compound are the same as described in the legend to fig 3.

lung, and heart from rats given ^{14}C-2-AAFC were significantly less than unity. In contrast, with the liver and kidney the rate of elimination from these organs was slower than that in the plasma with the result that by 4 hours each had substantially greater concentrations of drug than found in the plasma (figs 3 and 4). The high retention in the heart was conspicuous. As in animals given ^{14}C-2-AFC, the thymus and small intestine also appeared to retain substantial quantities of drug (*see* below). Finally, the DF values in the brain were minimal and only about one tenth of those found in rats given ^{14}C-2-AFC (fig 4). Many of the initial values in the brain may have been due to drug present in cerebral blood.

Retention by cardiac tissue as described above in rats was also noted in two dogs that were killed 4 hours after receiving iv injections of ^{14}C-2-AAFC (20 mg/kg). In one dog, the DF values found in the left ventricle, right ventricle, left auricle, right auricle, and plasma were 0.94, 0.99, 1.15, 0.95, and 0.30 respectively. In the other dog, the values were 0.67, 0.67, 0.67, 0.47, and 0.08 respectively.

By using a sensitive microbiologic assay, Neil and co-workers (16) measured plasma levels of ara-C and anhydro-ara-C in mice after ip injection. The elimination kinetics of ara-C and anhydro-ara-C during the first 6 hours in plasma appeared similar to those presented herein for AFC and AAFC respectively.

Conversion of AAFC to Active Metabolite

It has been proposed that AAFC and anhydro-ara-C are hydrolyzed to AFC and ara-C respectively

before being activated by phosphorylation (17,18). We have carried out preliminary studies to determine whether AAFC is, in fact, converted into nucleotides in vivo. The studies were also done to determine whether the prolonged retention of radioactivity in the heart, thymus, and small intestine of animals given ^{14}C-2-AAFC (figs 3 and 4) would be due to intracellular trapping of AFC-containing nucleotides. The results obtained are summarized in table 2. Consistent with earlier data from mice given tritiated ara-C (12), rats given ^{14}C-2-AAFC showed higher levels and longer retention of radioactivity in nucleotide fractions in the small intestine and thymus than in other tissues. A substantial portion of the radioactivity retained at 4 hours in the intestine was present as nucleotides. In the heart and thymus, however, most of the radioactivity was present in the nucleoside fraction, ie, in substances not bound by anion-exchange resin (*see* Materials and Methods). It is likely that the major constituent of the nucleoside fraction of the heart was unchanged AAFC since, as shown in figure 3, AFC is not retained by this organ. The reason for the presence of substantial amounts of radioactivity in the nucleoside fraction of thymus in rats receiving either labeled AFC or AAFC (fig 4) remains to be elucidated.

Hydrolyzed AAFC and its Biologic Effects

During complete hydrolysis of AAFC, less than molar equivalent amounts of AFC appeared (*see* pH 7.69 curves, fig 5); about 13% of the initial AAFC was converted to substances that had no ultraviolet absorbance in 5% perchloric acid due presumably to degradation of the pyrimidine ring. One degradation product was subsequently shown by Fox and Otter (10) to be fluoromalonaldehydic acid (fig 1), which could be expected to undergo conversion to monofluoromalonic acid and ultimately to monofluoroacetate. The latter compounds are known to block the Krebs cycle through inhibition of aconitase (19) and they are acutely toxic substances (20–22). The hall-

TABLE 2.—Distribution of metabolites in nucleoside and nucleotide fractions of rat tissues after iv injection of ^{14}C-2-AAFC (9 mg, 73 microcuries/kg)*

	μg of AAFC equivalents/g of tissue			
	1 hr		4 hrs	
Tissues	Nucleoside	Nucleotide	Nucleoside	Nucleotide
Small intestine	8.81	0.66	0.59 ± 0.01	0.64 ± 0.09
Liver	2.63	0.34	0.31 ± 0.09	0.038 ± 0.004
Thymus	2.31	0.21	1.28 ± 0.58	0.26 ± 0.08
Spleen	0.85	0.031	0.18 ± 0.02	0.032 ± 0.007
Heart	2.26	0.048	1.51 ± 0.18	0.062 ± 0.002
Lung	2.54	0.048	0.65 ± 0.15	0.056 ± 0.001
Kidney	6.68	0.20	0.50 ± 0.08	0.097 ± 0.010

*Rats were killed at 1 or 4 hrs after iv injection of ^{14}C-2-AAFC. Tissues were homogenized with 10% cold perchloric acid. The acid-soluble extracts, after removing perchloric acid as a potassium salt, were separated into nucleoside and nucleotide fractions (*see* Materials and Methods). The data at 1 hr are from a single animal and at 2 hrs from a pair of animals.

TABLE 3.—Toxicity of AAFC and hydrolyzed AAFC given ip to male CD rats

AAFC			Hydrolyzed AAFC		
Dose (mg/kg/day)	No. of daily doses	No. of deaths/ No. of rats*	Dose (AAFC equivalents in mg/kg)	No. of injections	No. of deaths/No. of rats*
250	5	0/6	211	1	0/6
500	5	0/6	421	1	4/7†
500	10	0/7	842	1	6/6‡

*All survivors were observed for 4 wks after injection.
†Death occurred in 3 rats between 2 and 5 hrs after injection and in 1 rat in <20 hrs.
‡Death occurred 40 mins to 4.5 hrs after injection.

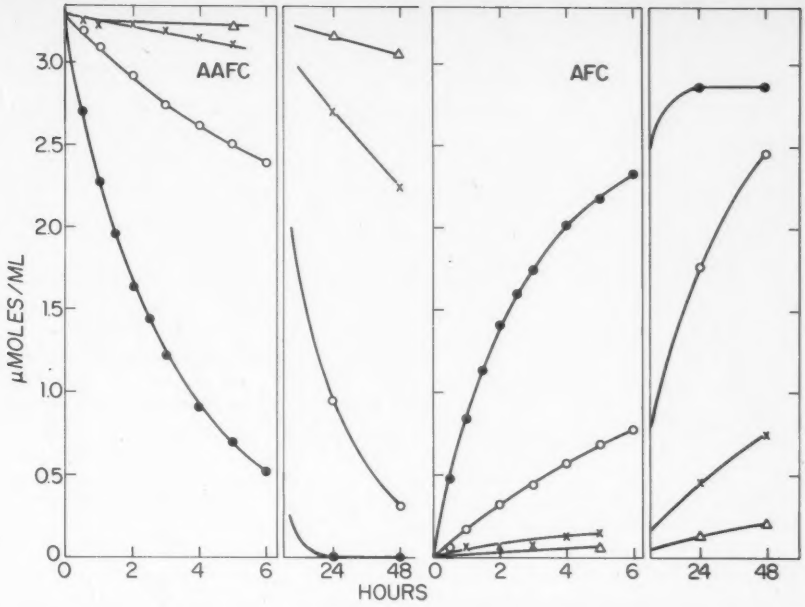

FIGURE 5.—Hydrolysis of AAFC at different pHs. 1 volume of sodium phosphate buffer, 0.5 M, to provide final pH values of 5.90, 6.83, or 7.69, or 1 volume of distilled water for a final pH of 5.80 was added to 10 volumes of AAFC (1 mg/ml). The mixtures were incubated at 37°C; after different time intervals, 1 ml of each mixture was added to 20 ml of 5% perchloric acid. Ultraviolet absorption spectra were determined in a Cary model 11 spectrophotometer. Concentrations of AFC were calculated for the absorbance of 300 nm where the extinction coefficient of AAFC is negligible. The values obtained were used to correct the absorbance at 268 nm, the maximum for AAFC. The corrected value was used to calculate the concentration of AAFC. Spectra of 24- and 48-hr hydrolysates at pH 7.69 were identical with that of authentic AFC. ● = pH 7.69; ○ = pH 6.83; × = pH 5.90; △ = pH 5.80.

mark of their action is the presence of high concentrations of citrate in tissues (19).

A comparison of toxicity of AAFC with hydrolyzed AAFC showed that the products of hydrolysis are, indeed, more toxic to rats than AAFC (table 3). The increased toxicity was not due to AFC since rats survive doses of this substance as large as 800 mg/kg/day given ip for 5 successive days. The lethal effects of hydrolyzed AAFC developed rapidly. The animals became depressed, ataxic, dyspneic, and tremorous and had episodes of clonic convulsions. Most deaths occurred within < 5 hours after injection. Such acute changes have not been seen in rats treated with either AAFC or AFC. They did, however, resemble the acute lethal responses of rats to monofluoroacetic acid or monofluoromalonic acid (20–22). It seemed reasonable to attribute them to the formation of fluoromalonaldehydic acid that occurs during hydrolysis of AAFC (*see* above).

Further evidence for the formation of a hydrolytic product with fluoroacetate-like properties was found in measurements of citrate concentrations measured in organs of rats given AAFC or hydrolysates of AAFC. As shown in table 4, the injection of these substances induced marked increases in citrate in the kidney, heart, liver, brain, and muscle. No such accumulations of citrate took place in rats given AFC.

The retention of AAFC by the heart, which has been described above, suggests the possibility that hydrolysis of the substance in situ could result in the formation of fluoroacetate-like derivatives in relatively high concentrations in cardiac tissue. Accordingly, AAFC might be expected to have cardiotoxic effects since one of the primary sites of toxic action of fluoroacetate is in cardiac muscle (20,21). In unpublished observations by Dr. A. Gilladoga, we have found that AAFC does, in fact, induce arrhythmias and cardiac failure in dogs. In phase I clinical studies of AAFC, electrocardiographic abnormalities and arrhythmias have been noted in a few patients (8).

TABLE 4.—Citrate in rat tissues 4 hrs after ip injection of AAFC (1000 mg/kg), hydrolyzed AAFC (218 mg/kg), AFC (1000 mg/kg), and saline*

Agent injected	No. of rats	Tissue				
		Heart	Liver	Kidney	Muscle	Brain
AAFC	4	1070 ± 92	782 ± 97	1551 ± 104	53 ± 6	112 ± 22
Hydrolyzed AAFC	4	784 ± 63	151 ± 110	676 ± 62	35 ± 5	149 ± 20
AFC	3	32 ± 2	17 ± 3	32 ± 2	20 ± 1	—
Saline	7	27 ± 1	14 ± 1	28 ± 3	20 ± 2	30 ± 2

*4 rats were used. The data are given in μg citric acid/g of tissue.

LIST OF COMPOUNDS

AFC: NSC-529180; CAS reg. No. 4298-10-6; 1-β-D-arabinofuranosyl-5-fluorocytosine

AAFC: NSC-166641; CAS reg. No. 40505-45-1; 2,2'-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine, monohydrochloride

AFU: NSC-406444; CAS reg. No. 131-06-6; 1-β-D-arabinofuranosyl-5-fluorouracil

Anhydro-ara-C: NSC-129220; CAS reg. No. 31698-14-3; 2,2'-anhydro-1-β-D-arabinofuranosylcytosine, monohydrochloride

Ara-U: NSC-68928; uracil, 1-β-D-arabinofuranosyl-

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

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Methotrexate, Cytosine Arabinoside, and BCNU Concentration in Brain After Ventriculocisternal Perfusion[1]

Ronald G. Blasberg[2,3,*]

SUMMARY

The movement of drug from cerebrospinal fluid (CSF) across the ependymal or pia-glial surface into and through the brain is an important consideration if we hope to better understand the pharmacologic effects of intrathecal drug administration. From an analysis of the periventricular brain tissue profile (drug concentration versus distance from the ependymal surface) after ventriculocisternal perfusion, it is possible to measure the brain tissue concentration of drug, the apparent diffusion constant, or the capillary exchange half-life of the drug in brain. This technique was used to study the CSF-to-brain transfer of five chemotherapeutic drugs in the rhesus monkey. Methotrexate (MTX) and hydroxyurea were similar; they crossed the ependyma but did not readily exchange across brain capillaries. They moved through the brain extracellular fluid (ECF) by diffusion, and an apparent diffusion constant in brain could be determined which was 1.2 and 2.0 $\times$ 10^{-6} cm^2/second, respectively, for the two drugs. Cytosine arabinoside crossed the ependyma and was continually accumulated by brain cells; this reduced the amount of drug in the ECF and effectively diminished drug penetration further into the brain. Cytosine arabinoside does not readily exchange across brain capillaries; its apparent diffusion constant in brain could not be determined from these studies, but it probably is less than that of MTX and hydroxyurea. BCNU and thioTEPA cross the ependyma and exchange rapidly across brain capillaries; a capillary exchange half-life could be determined which was 0.8 and 1.0 minutes, respectively. Post-perfusion periventricular concentration profiles for MTX were computed and are presented graphically; they demonstrate the importance of drug clearance from the CSF. Knowledge of the apparent diffusion constant and the capillary exchange half-life of a drug in brain and the rate of drug clearance from the CSF is useful in planning a more rational schedule for intrathecal drug administration.

[Cancer Treat Rep 61:625–631, 1977]

Most drugs are administered orally or iv and are delivered to the brain by the systemic circulation. The parameters which affect the delivery and distribution of a drug to the brain after systemic administration have already been well discussed at this Workshop. There are certain clinical situations, however, when it appears more advantageous to administer a drug into the cerebrospinal fluid (CSF). Significantly higher CSF concentrations of certain drugs may be obtained after direct intrathecal administration than after systemic administration. Drug distribution within and clearance from various CSF compartments after intrathecal push administration are important parameters affecting drug delivery, but they will not be considered here. This discussion will focus on the movement of drug from CSF across the ependymal or pia-glial surface into the brain.

[1] Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

BCNU: NSC-409962; CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

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[3] Many of the ideas expressed in this manuscript had their origin in Dr. David P. Rall's laboratory (NCI) in the early 1960s; continuation of this work is being performed in Dr. Joseph Fenstermacher's laboratory (DCT, NCI). Mathematic analysis was provided by Dr. Clifford S. Patlak (National Institute of Mental Health, National Institutes of Health [NIH]).

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A schematic diagram of drug movement from CSF into and through the brain is presented in figure 1. The first series of arrows represents the movement of a compound from the CSF through the brain extracellular fluid (ECF); little or no cellular uptake and capillary exchange occurs. The driving force of solute or drug movement from CSF across the ependyma and through the brain ECF is diffusion down a concentration gradient (high in the CSF to low in the brain). Diffusional movement through the brain may be augmented or retarded by bulk flow of entrained solute. Our studies in the rhesus monkey indicate that little or no bulk flow occurs and therefore it will not be discussed further. The compound represented by the first series of arrows (fig 1) would be an ideal extracellular space (ECS) marker; for other compounds or drugs with biologic activity, the pattern is more complex. In the second series of arrows, the drug readily enters and equilibrates with the surrounding cells and may even cross the capillary endothelium to a limited extent. The net effect of cellular uptake will be to reduce the amount of drug in the ECF and thereby reduce the amount of drug that is available to diffuse deeper into the brain as illustrated by a smaller arrow. Similarly, in the third series of arrows, we see the drug readily crossing the capillary endothelium as well as readily entering and equilibrating with cells. Drug which crosses the capillary endothelium enters the blood and systemic circulation. The drug is lost from the ECF as well as from the entire brain. If the transcapillary exchange is sufficiently rapid, a steady state is soon reached in which the amount of drug diffusing from the CSF into and through the brain is balanced by an equal amount of drug diffusing across the capillaries into the systemic circulation.

A convenient way to study the movement of drug from CSF to brain is to perform a ventriculocisternal or ventriculolumbar perfusion. A constant concentration of drug is maintained along the perfused surface of the brain during which time drug diffuses into and through the brain. Animal studies permit the sampling of periventricular brain tissue. Serial slices of a rectangular block of tissue cut perpendicular to the perfused ependymal surface can be obtained with a freezing microtome, and the drug concentration in each tissue slice can be assayed. A tissue profile of drug concentration versus distance from the perfused ependymal surface can be constructed. The details of the tissue concentration profile analysis are presented elsewhere; suffice it to say that a measure of the apparent diffusion constant or the half-life of ECF-transcapillary exchange of a particular drug in brain may be determined (1–3).

We have studied the movement of five chemotherapeutic agents from CSF into brain in the rhesus

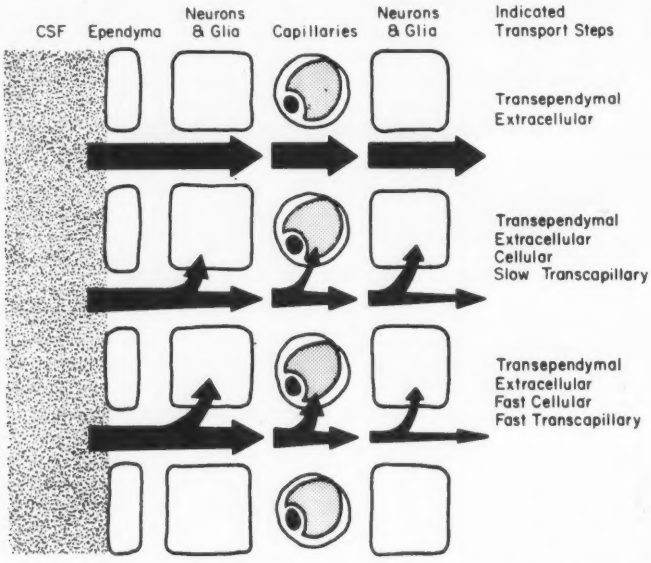

FIGURE 1.—Schematic representation of several possible transport pathways into and through the brain for a drug that has been introduced into the ventricular spinal fluid. Arrows represent the pathways and thickness of the arrows indicates the relative importance of each pathway.

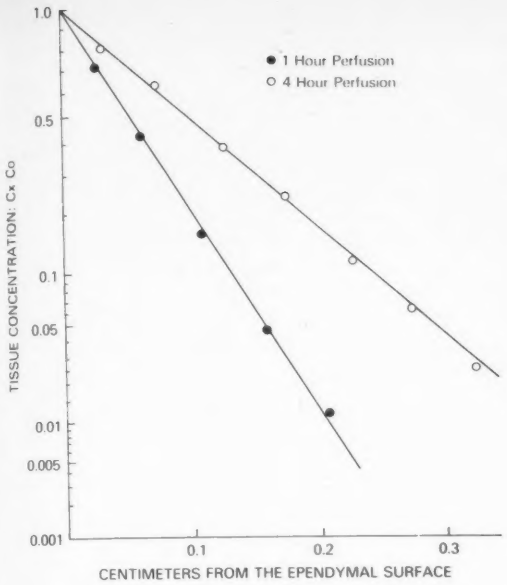

FIGURE 2.—Inverse complimentary error function graph presenting the periventricular tissue concentration profiles of MTX after 1- and 4-hr ventriculocisternal perfusion. Tissue concentration (C_x) which was measured experimentally at each point has been normalized to the subependymal concentration (C_o) and is expressed as a ratio (C_x/C_o).

monkey (4). The five drugs were methotrexate (MTX), cytosine arabinoside, hydroxyurea, BCNU, and thioTEPA. The movement of MTX and hydroxyurea demonstrated similar characteristics as did the movement of BCNU and thioTEPA. Therefore, only MTX, cytosine arabinoside, and BCNU will be discussed.

MTX

The tissue concentration profiles of MTX in the caudate nucleus after a 1- and a 4-hour perfusion are presented in figure 2. The longer the perfusion, the deeper MTX penetrates into the brain. However, the depth of penetration increases with the square root of the perfusion time; that is, it takes four times the initial perfusion time to double the initial depth of drug penetration. The apparent diffusion constant of the drug in brain may be calculated from the slope of the drug concentration profiles in figure 2. The subependymal tissue space and the apparent diffusion constant of MTX in brain are presented in table 1.

How is this information helpful to us? Once the apparent diffusion constant of a drug in the brain is known, it is possible to estimate the length of ventriculocisternal or ventriculolumbar perfusion necessary to establish a certain concentration of drug at a particular distance within the brain. Another way of asking the question is, what administration schedule is necessary to achieve a given concentration of drug at a specific point within the brain? These calculations were performed and they are presented in table 2.[1]

TABLE 1.—Brain tissue space, diffusion constant, and ECF-transcapillary exchange half-life determined by ventriculocisternal perfusion

Drug	Tissue space*	Diffusion constant (cm^2/sec)	Capillary half-life (mins)
MTX	27	1.2×10^{-6}	> 360
Cytosine arabinoside	> 170	—	> 360
BCNU	64	$\simeq 14 \times 10^{-6}$	0.8

*cpm/mg tissue ÷ cpm/μl of perfusate at CSF-brain interface (C_o).

The drug concentration we arbitrarily chose to achieve was 10% of its perfusate (or CSF) concentration for two distances, 0.1 and 1.0 cm, into the brain from the ependymal surface. The calculations use the MTX diffusion constant determined in brain caudate nucleus from the 1-hour experiments, 1.2×10^{-6} cm^2/second. A perfusion of only 26 minutes is required to reach one tenth of the perfusate (or CSF) concentration ($C_x/C_o = 0.1$) at a distance 0.1 cm into the brain (*see* column 2, table 2). However, to reach the same concentration at a depth of 1.0 cm into the brain, a perfusion of 43 hours is required. What is immediately apparent from these calculations is that diffusion is an effective means for the transport of MTX over short distances in the range of ≤ 0.1 cm but a poor mode of transport over longer distances in the range of ≥ 1.0 cm.

If an acceptable brain ECF concentration is only 1% ($C_x/C_o = 0.01$) or 0.1% ($C_x/C_o = 0.001$) of the perfusate (or CSF) concentration, a somewhat different picture emerges. The same calculations were performed and are presented in the third and fourth columns, respectively, of table 2. Considerably shorter perfusions are required to establish the lower tissue ECF MTX concentrations. However, we must still think in terms of hours for MTX to diffuse

[1]The calculations assume that the brain capillaries are impermeable to MTX and that MTX is not metabolized during the perfusion period; diffusion is considered along rectangular coordinates into an infinitely thick slab of brain tissue. Another paper in this Workshop (ref 5) deals with the effects of capillary permeability, metabolism, and more realistic brain tissue diffusion distances compatible with the human brain.

TABLE 2.—Calculated perfusion times required to reach a particular brain ECF concentration (C_x/C_o) at a particular distance (x) from the perfused surface for MTX*

Distance (x)	Calculated perfusion time		
	$C_x/C_o = 0.1$	$C_x/C_o = 0.01$	$C_x/C_o = 0.001$
0.1 cm	26 mins	11 mins	6 mins
1.0 cm	43 hrs	17 hrs	11 hrs

*$C_x/C_o = \text{erfc} \frac{x}{2\sqrt{Dt}}$, where D = diffusion constant and t = length of perfusion.

≅ 1.0 cm into the brain from the perfused surface, even if an acceptable ECF tissue concentration is three log orders below that in the ventriculocisternal perfusate (or CSF).

Cytosine Arabinoside

The tissue concentration profiles of cytosine arabinoside after a 1- and a 4-hour perfusion are presented in figure 3. A steady state was not reached by 1 hour; drug continued to be accumulated by the tissue resulting in a significantly higher concentration after 4 hours of perfusion. A comparison of data in table 1 and figures 1 and 2 demonstrates that although cytosine arabinoside is accumulated by the periependymal tissue to a far greater extent than MTX, it does not move as readily into the brain. This difference cannot be due to the slight difference of their aqueous diffusion constants: 8.5 and 5.3 cm^2/second × 10^{-6} at 37°C for cytosine arabinoside and MTX, respectively. Since both drugs must diffuse through the same ECS pathways and have similar diffusion constants in water, the slower movement of cytosine arabinoside into the brain is due to the greater uptake of cytosine arabinoside from the ECF by brain cells. The relative concentration of cytosine arabinoside in the ECF is less than

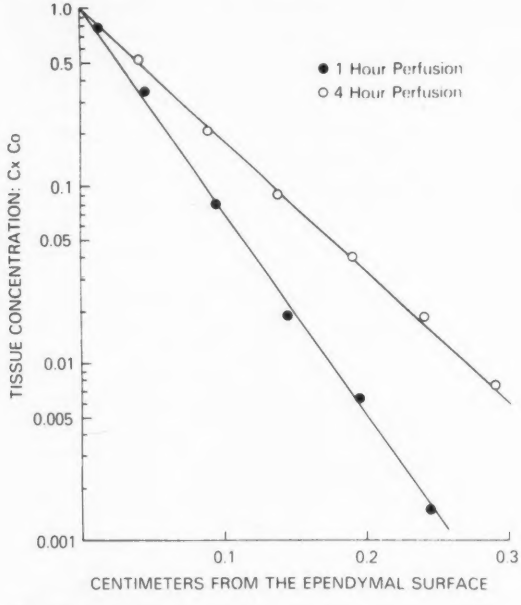

FIGURE 3.—Semilog graph presenting the periventricular tissue concentration profiles of cytosine arabinoside after 1- and 4-hr ventriculocisternal perfusion.

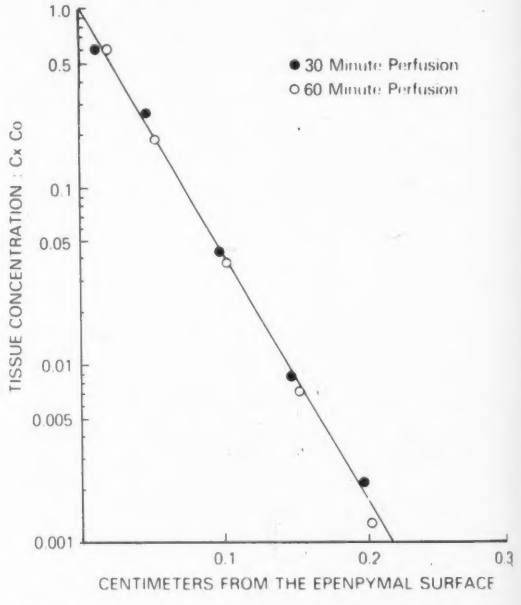

FIGURE 4.—Semilog graph presenting the periventricular tissue concentration profiles of BCNU after 30- and 60-min ventriculocisternal perfusion.

that of MTX; therefore less cytosine arabinoside is available to diffuse further into the brain.

The apparent diffusion constant for cytosine arabinoside in brain could not be determined from these experiments because the analysis requires that the subependymal tissue be at a steady state. However, it is clear that the apparent diffusion constant of cytosine arabinoside through the brain ECF is less than that of MTX. The movement into and through the brain of cytosine arabinoside would be schematically represented by the second row of arrows in figure 1, whereas the movement of MTX would be represented somewhere between the first and second row of arrows in the same figure.

The previous discussion concerning MTX and cytosine arabinoside only applies to drugs which do not cross brain capillaries readily, that is the so-called polar, non-lipid-soluble class of drugs. If we now consider the non-polar, lipid-soluble drugs, a different pattern emerges. These drugs exchange readily across brain capillaries. They will diffuse from CSF into and through the brain down a relatively steep concentration gradient. This steep concentration gradient is maintained by the diffusion of drug from the brain ECF across brain capillary endothelial cells into the systemic circulation. This pattern was observed with two of the drugs we studied, BCNU and thioTEPA, and is schematically represented by the third row of arrows in figure 1.

BCNU

The tissue concentration profiles of BCNU after a 30-minute and a 1-hour perfusion are presented in figure 4. The tissue concentration profiles are identical. In contrast to MTX and cytosine arabinoside, increasing the perfusion time did not increase the depth of BCNU penetration into the brain. The tissue has reached a steady state by 30 minutes. The amount of BCNU diffusing from CSF into and through the brain is balanced by an equal amount of BCNU diffusing across the capillary endothelium into the systemic circulation. A capillary exchange half-life may be calculated from the slope of the tissue concentration profile. The capillary exchange half-life for BCNU is presented in table 1. The exchange half-life is very short, indicating that the capillary transfer constant is high and that the capillaries are permeable to this drug.

It is apparent that little drug reaches beyond the first several millimeters of periependymal brain tissue when a non-polar lipophilic drug is administered by way of the CSF. The tissue concentration profile remains steep as does the diffusion gradient from CSF into and through the brain.

TABLE 3.—Drug clearance from perfusate during 1-hr ventriculocisternal perfusion

Drug	Perfusate clearance* (μl/min)	Fraction of bulk CSF clearance†
EDTA-Ca	4	0.11
MTX	16	0.44
Cytosine arabinoside	22	0.61
BCNU	145	4.0

*Perfusate clearance was calculated by the equation of Heisey et al (ref 9) using a constant perfusion rate of 197 μl/min and an average CSF production rate of 36 μl/min.

†Fraction of bulk CSF clearance equals the ratio of perfusate clearance over the average CSF production rate; CSF production rate is assumed to be equal to bulk CSF absorption or clearance rate.

Ventriculocisternal Perfusate Clearance

The movement of BCNU, or any other lipid-soluble drug, from the CSF into the brain and subsequent clearance by brain capillaries may account for a significant fraction of total CSF clearance. This is seen in table 3. Ethylenediamine tetraacetic acid-calcium (EDTA-Ca) is a reference compound and is cleared slowly. MTX and cytosine arabinoside are cleared somewhat more rapidly than the reference compound which probably reflects their cellular uptake by periventricular brain tissue and/or uptake and clearance by the choroid plexus. The non-polar, lipid-soluble drugs like BCNU are cleared very rapidly from the ventriculocisternal perfusate. This is consistent with their high capillary transfer constants. Therefore, diffusion from the CSF into the brain will account for a significant fraction of CSF clearance if the brain capillary transfer constants are high.

Post-Perfusion Periventricular Tissue ECS Concentration Profile

Following the cessation of ventriculocisternal or ventriculolumbar perfusion, the concentration of drug in the CSF will fall as the drug is being cleared. During this period, drug will continue to diffuse through the ECF down its concentration gradient deeper into the brain parenchyma. If the fall in CSF concentration is sufficiently rapid, the concentration of drug in the ECF of periependymal or peri-pia-glial brain tissue may exceed that in the adjacent CSF. Drug will then diffuse from the brain back into the CSF and will continue to diffuse further into the brain. These post-perfusion events

have been computed for MTX and are presented graphically in figure 5.[5]

All three graphs illustrate a ventriculocisternal perfusion with MTX for 1 hour. The periventricular MTX profile after the 1-hour perfusion is shown by the slightly heavier straight line intercepting the ordinate at C_x/C_o = 1.0. Following the 1-hour MTX perfusion, the ventricular MTX concentration was set at 0 on the left graph (as though the ventricular CSF was being rapidly cleared by a rapid perfusion of drug-free CSF) in order to magnify the concentration gradient between periventricular brain tissue ECF and adjacent CSF. In the center and right graphs, the post-perfusion ventricular MTX concentrations were established by assuming a CSF-MTX clearance half-life of 1 and 6 hours, respectively. The ordinate intercept reflects the ventricular CSF-MTX concentration at each post-perfusion time.

The post-perfusion concentration profiles of MTX in figure 5 clearly demonstrate the points outlined above. The periventricular brain tissue levels of MTX fall with increasing post-perfusion time. MTX diffusional transport in the brain is occurring in two directions: from the region of maximum brain concentration to the CSF as well as to deeper brain regions with comparatively low MTX concentrations. Although the maximum brain concentration falls with increasing post-perfusion time, the peak concentration moves further into the brain and the concentration gradient on both sides of the peak becomes less steep.

The most important observation is that by decreasing the rate of MTX clearance from the ventricular CSF (by moving left to right in fig 5), considerably higher concentrations of MTX are maintained in the brain and higher levels are reached in deeper brain tissue for a longer period of time. Considerably more drug enters and remains in the brain when MTX clearance from the CSF is slow in contrast to the considerable back flux of MTX from brain to CSF when the clearance of drug from the CSF is high. There are two ways to reduce MTX clearance from the CSF: (*a*) block the choroidal uptake and clearance of MTX with transport inhibi-

[5]*See* footnote 4; the same assumptions apply to the simulated post-perfusion tissue concentration profiles. The calculations were performed by Dr. Clifford S. Patlak.

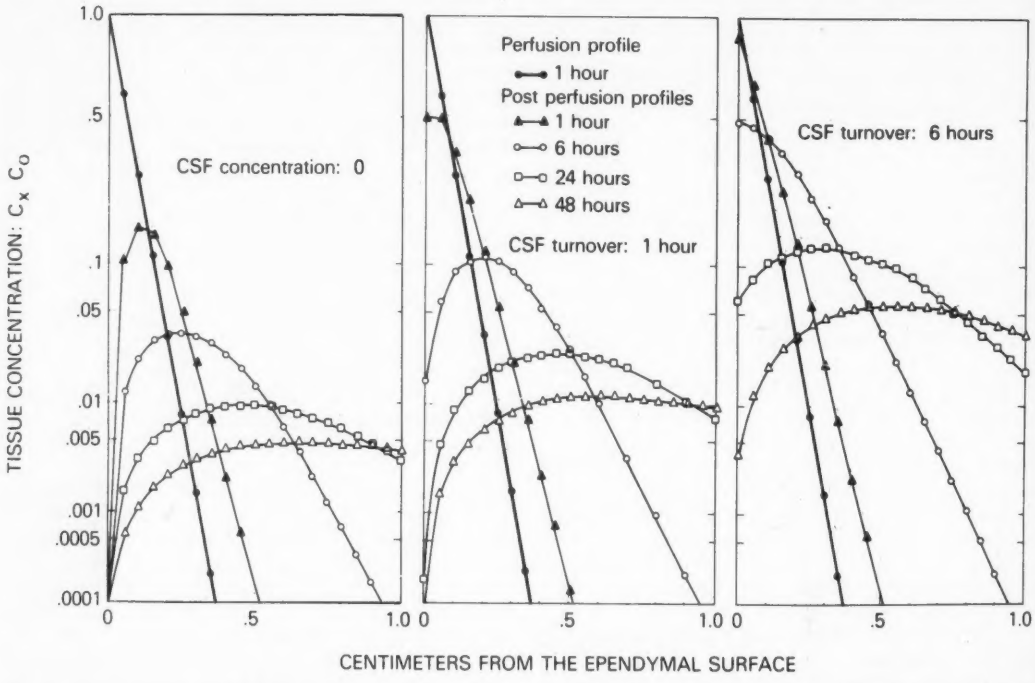

FIGURE 5.—3 inverse complimentary error function graphs presenting a graphic simulation of the post-perfusion concentration profiles of MTX in periventricular brain tissue. Tissue ECF concentration (C_x) is normalized to the subependymal ECF concentration (C_o) and is expressed as a ratio (C_x/C_o).

tors such as probenicid, and (*b*) reduce bulk CSF turnover or clearance by reducing CSF production with acetazolamide or cardiac glycosides (6).[6] Such techniques may double or triple the clearance half-life of MTX from the CSF and would have a profound effect on the brain ECS concentration of MTX. In the case of a partial or complete obstruction of CSF flow (due to an aqueductal, cisternal, or subarachnoid block caused by tumor, adhesions, or congenital abnormality), prolonged CSF clearance half-lives of MTX (far > 6 hours) may be obtained which could result in a prolonged elevation of periventricular and/or peri-pia-glial tissue drug levels. Such conditions could well account for some of the neurotoxic effects and the leukoencephalopathy that has been observed (7). This consideration becomes all the more plausible when one considers that the antifolate effects of MTX are observed at concentrations of three log orders or more below the initial CSF concentration following intrathecal or intraventricular administration (8).

Drug Metabolism

The previous discussion assumes that the drug remains unaltered in its administered form. The experimental data from which we calculated the apparent diffusion constants and the capillary exchange half-lives in brain are based on drug-derived radioactivity. In the case of BCNU, some of the radioactivity we measured after a 1-hour ventriculocisternal perfusion was from a BCNU metabolite. Drug metabolism in the brain to either an active, inactive, or toxic product must be considered differently with somewhat more complex mathematics. Insofar as one is interested only in the administered form of the drug, drug metabolism may be viewed as we have viewed capillary exchange. Drug metabolism reduces the amount of the administered form of the drug in the ECF; less drug is available to diffuse further into the brain. Therefore, drug metabolism will effectively reduce the penetration of the administered form of the drug into the brain.

[6]Spector R. Modification of folate and methotrexate between blood and CSF. Presented at Workshop on Antimetabolites and the Central Nervous System, New York, NY, Feb 2–3, 1976.

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Distribution of Methotrexate in the Cerebrospinal Fluid and Brain After Intraventricular Administration [1]

Ronald G. Blasberg,[2,*] Clifford S. Patlak,[3] and William R. Shapiro [4]

SUMMARY

There are several parameters which significantly affect methotrexate distribution in the cerebrospinal fluid (CSF) and in the brain extracellular fluid (ECF) after intraventricular or intralumbar administration. These parameters are developed through the correlation of an experimental study, which analyzed the periventricular concentration of methotrexate after ventriculocisternal perfusion, with a clinical study of methotrexate pharmacodynamics in CSF and serum after intraventricular administration. A model of methotrexate flow dynamics in the CSF after intraventricular administration was constructed; a computer fit of the observed data points indicates that there is a rapid flux between ventricular and basal cistern CSF compartments and a comparatively slow flux between ventricular (or basal cistern) and lumbar sac CSF compartments. Only a very small fraction of the methotrexate administered appears to penetrate the brain and/or to be cleared by the choroid plexus; the back flux of methotrexate from the brain to the CSF does not appear to significantly affect the CSF concentration. The "reservoir" effect of CSF methotrexate and the apparent rate-limiting effect of CSF clearance on serum clearance are discussed. Periventricular ECF concentration of methotrexate as a function of time was computed and is presented graphically. The graphic analysis indicates that diffusional transport of methotrexate to periventricular or peri-pia-glial brain tissue is rapid but it is a very slow means of transport to deep brain tissue. Methotrexate concentrations in the brain can be increased by (*a*) reducing the rate of methotrexate clearance from the CSF; (*b*) distributing methotrexate throughout the CSF so diffusional transport is occurring from opposing CSF surfaces; and (*c*) multiple-dose scheduling. Diffusional transport through the ECF will be retarded and brain concentrations of methotrexate will be reduced by transcapillary loss.

[Cancer Treat Rep 61:633–641, 1977]

This paper will attempt to correlate some of our experimental data and mathematic analyses of methotrexate distribution in the brain during and after ventriculocisternal perfusion with recent clinical data on methotrexate pharmacodynamics after both systemic and intraventricular administration (1,2). This correlation will offer some predictive guidelines and define certain limits for methotrexate delivery to the brain by way of the cerebrospinal fluid (CSF). Hopefully, the design of future intrathecal chemotherapy protocols utilizing methotrexate (or any other drug) will benefit from this review.

Physical Characteristics

Methotrexate is a weak organic acid with a molecular weight of 456. It has a pKa value of 4.7 and is slightly soluble in water at physiologic pH (1.6 mg/cm^3 at 25°C). It is very slightly soluble in alcohol and insoluble in organic solvents. The log of the partition coefficient (log octanol/water) is −1.85. Metho-

[1] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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trexate is 20%–50% bound to serum albumin over a fairly wide concentration range in the blood.[5] The diffusion constant of methotrexate in water is 5.3×10^{-6} cm²/second at 37°C. For comparative purposes, the aqueous diffusion constants of sodium and serum albumin are 20×10^{-6} and $1–2 \times 10^{-6}$ cm²/second respectively at 37°C. The physical characteristics of methotrexate largely account for its distribution in the central nervous system (CNS) as well as the relative exclusion of this molecule from the CNS after systemic administration.

Systemic Administration

Experimental and clinical studies have repeatedly demonstrated that CSF methotrexate levels remain significantly below plasma levels after systemic administration (3,4). In a recent clinical study by Shapiro et al (2), serial serum and ventricular CSF methotrexate levels were determined during and following a constant iv infusion of methotrexate. Some of those data are shown in figure 1. Serum methotrexate levels remained relatively constant at slightly $> 10^{-5}$ M. Ventricular CSF levels rose rapidly with an equilibration half-time between 4 and 6 hours. After equilibration, however, ventricular methotrexate levels were only 3%–5% of serum levels. The binding of methotrexate to serum proteins will account for a significant part of the concentration gradient between serum and CSF.

Following cessation of the iv infusion, serum methotrexate levels decreased rapidly with an initial serum clearance half-time of 4 hours. By 36 hours, serum methotrexate levels were $< 10^{-6}$ M. The subsequent decrease in serum methotrexate was considerably slower and probably reflects a back flux of drug from extravascular tissue compartments. Ventricular methotrexate levels, after cessation of the iv infusion, remained below serum levels although the initial fall in ventricular methotrexate was not as rapid as that in the serum. The ventricular CSF clearance half-time, given the wide range in the data points, was approximately 6 hours.

Our review of the literature indicates that there have been no published studies which have measured both brain and CSF concentrations of methotrexate after an equilibration period during which time the serum methotrexate concentration has been maintained constant. Therefore, it would be unwise at this time to extrapolate from the CSF concentration of methotrexate to the brain concentration of methotrexate, particularly after a single iv injection when serum levels are changing rapidly.

[5] Chabner BA. Unpublished results.

Intraventricular Administration

The distribution of methotrexate within the CSF after intrathecal administration is dependent on a number of factors: (*a*) site and mode of administration; (*b*) mixing and bulk movement; (*c*) bulk clearance; (*d*) choroidal uptake and clearance; and (*e*) movement across the CSF-brain border. Local mixing of drug within a CSF compartment is largely due to local turbulence generated by choroidal and arterial pulsations and to the effects of ependymal cilia. The movement of drug from one CSF compartment to another or the equilibration of drug between CSF compartments is largely due to the dynamics of bulk CSF movement as well as the choroidal and arterial pulsations. Diffusion does not contribute significantly to drug distribution between CSF compartments because the diffusion distances between compartments are long. Drug clearance is largely due to bulk CSF clearance of entrained drug as well as to choroidal uptake and clearance and to the movement of drug across the CSF-brain border. Methotrexate is taken up by choroid plexus in vitro (5); choroidal uptake in vitro and the clearance of methotrexate from CSF in vivo appear to involve a probenecid-sensitive transport system.[6] During ventriculocisternal perfusion studies in the rhesus monkey, methotrexate clearance was observed to be 16 μl/minute (1). This was approximately 44% of the CSF production rate. The clearance of methotrexate in these studies represents the sum of choroidal clearance plus diffusion into the periventricular brain parenchyma. Diffusion into the periventricular brain parenchyma may account for only a small fraction of methotrexate clearance since the clearance of an extracellular space (ECS) marker compound, EDTA-Ca, was only 4 μl/minute in these studies (1).

In the clinical study of Shapiro et al (2), serial ventricular and lumbar CSF and serum methotrexate concentrations were measured after a single intraventricular injection of methotrexate. The data are presented in figure 2. Over the first several hours, the ventricular concentration of methotrexate falls rapidly while the lumbar concentration rises rapidly. The cross-over point occurs between 4 and 6 hours after which time the lumbar concentration exceeds the ventricular concentration by fourfold to fivefold. The fall in ventricular methotrexate is not linear on the semilog plot. Therefore, methotrexate clearance from the ventricle cannot be described by a single exponential function. A model with more than one compartment is required to fit

[6] Spector R. Modification of folate and methotrexate between blood and CSF. Presented at the Workshop on Antimetabolites and the Central Nervous System, New York, Feb 2–3, 1976.

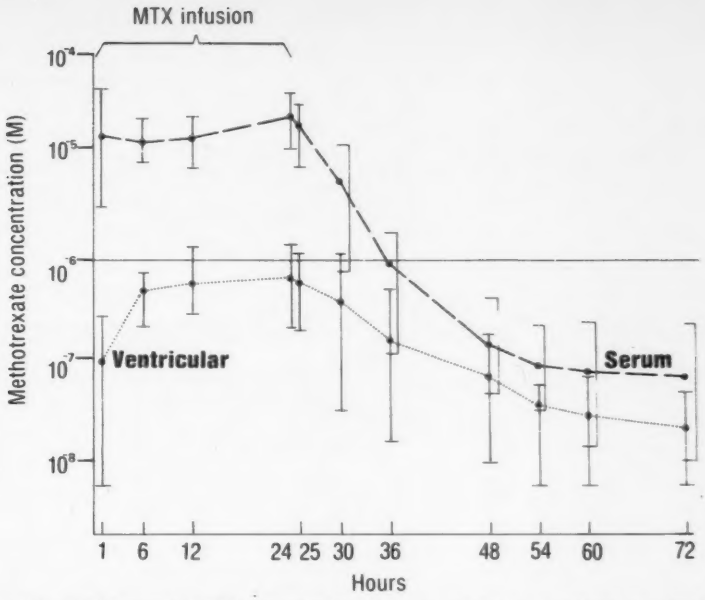


FIGURE 1.—Methotrexate (MTX) concentration (mean ± range) in 4 patients who received iv methotrexate (500 mg/m²) during a 24-hr infusion. (Reprinted by permission from the N Engl J Med 293:162, 1975.)

the data. Between 16 and 48 hours, however, the semilog plot of both ventricular and lumbar methotrexate concentration is reasonably linear and the approximate half-times of methotrexate clearance were calculated. A clearance half-time between 6 and 8 hours was obtained for both the ventricular and lumbar CSF compartments.

The serum methotrexate rise after intraventricular methotrexate administration is less rapid than the rise in the lumbar CSF; the serum concentration reaches a maximum by 8 hours. The subsequent fall in the serum methotrexate concentration has a clearance half-time of 12 hours which is considerably longer than that observed after iv administration. The prolonged serum clearance of methotrexate after intraventricular administration, in comparison to the serum clearance after the iv infusion of methotrexate calculated above, will reflect the longer clearance of methotrexate from the CSF compartment (into the vascular compartment) during the latter post-perfusion period. The "reservoir" effect of the brain and CSF and the rate-limiting clearance of methotrexate from the CSF compartment could well account for some of the systemic toxicity that has been observed after intrathecal methotrexate administration (6). The clearance half-times are summarized in table 1.

Model of Methotrexate Flow Dynamics

A simplified model of methotrexate flow dynamics in the CSF after intraventricular methotrexate administration is presented in figure 3. The model incorporates some of the parameters which have been discussed above. The k's represent flow constants (cm³/hour) since the ventricular and lumbar methotrexate data obtained in the study by Shapiro et al (fig 2) are expressed as concentrations; therefore, a volume term, V (cm³), has to be introduced into the differential equations. We had constructed several reasonable models of methotrexate flow dynamics in the CSF and were able to fit the observed data points with the aid of a computer program. However, an unsatisfactory fit of the last several ventricular and lumbar CSF data points was frequently obtained. The model appeared to require a periventricular brain compartment and/or a larger ventricular CSF compartment to obtain a good fit of the measured data points.

The calculated flow constants of methotrexate between ventricle and basal cistern (k_1 and k_2) were very high, indicating a rapid exchange between compartments 2 and 3. This suggests that the ventricular CSF has essentially equilibrated with the cisternal CSF by 1 hour. The 1-hour measurement

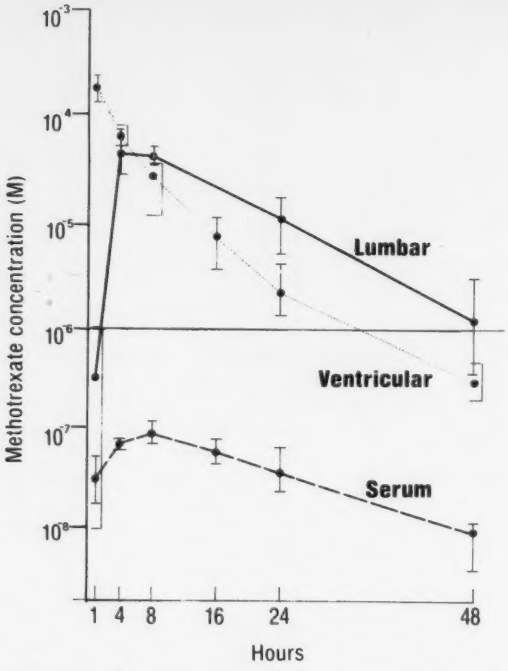


FIGURE 2.—Methotrexate distribution (mean ± range) in 5 studies after administration (6.25 mg/m²) via an Ommaya reservoir. (Reprinted by permission from the N Engl J Med 293:163, 1975.)

and subsequent measurements of ventricular methotrexate concentration could therefore be considered to reflect the methotrexate concentration of a single large well-stirred compartment. This single compartment would be composed of the ventricular and cisternal CSF. A two compartment model (combining the above compartments plus the lumbar compartment) was constructed and a good fit of the data points was obtained.

The periventricular tissue (compartment 1) did not appear to significantly affect methotrexate clearance from ventricular CSF. Much to our surprise, both the volume and the flux of drug (k_7, k_8, and k_9) into and out of compartment 1 were comparatively small. Only when the bulk clearance of methotrexate (k_6) was set 25% below the calculated bulk clearance of CSF (22 cm³/hour)[7] did the periventricular brain compartment and the clearance of methotrexate by the brain and choroid plexus (k_{41}) become significant. Good fits of the data could be

TABLE 1.—Methotrexate clearance after iv and intraventricular administration*

Compartment	Methotrexate clearance: half-time	
	Iv administration (hrs)	Intraventricular administration† (hrs)
Serum	4	12
Ventricular CSF	6	6–8
Lumbar CSF	—	7

*Clearance half-times were calculated from the data of Shapiro et al (2).

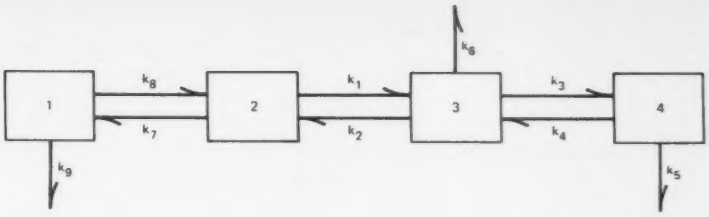
†Values determined from data points beyond 16 hrs.

obtained for values of the bulk clearance of methotrexate (k_6) varying between 17 and 22 cm²/hour. Several measurements of ventricular (and cisternal) methotrexate concentration during the first hour after administration would be necessary to more accurately define the flow constants (k_1, k_2, k_7, k_8, and k_9).

Several important points become apparent from the above analysis of data obtained by Shapiro et al (2). First, there is a very rapid flux or exchange of methotrexate between the ventricular and the basal cistern compartments in contrast to the comparatively slow flux or exchange of methotrexate between the ventricular (or basal cistern) and the lumbar sac. The flux from ventricle to cistern (k_1) was > 100 cm³/hour whereas the flux from basal cistern to lumbar sac (k_3) was 2.1 cm³/hour. A recent study has demonstrated that ^{131}I-serum albumin descends to the basal cistern within 5–10 minutes after intraventricular administration (8). Although cisternographic studies do not demonstrate isotope in the ventricles of normal individuals after intrathecal lumbar administration, significant activity is encountered if ventricular fluid is sampled directly.[8] Second, the periventricular brain compartment appears to be small. The data are not sufficient to quantitate small fluxes between small compartments. Third, the computed volumes for the basal cistern (V_3) and for the lumbar sac (V_4) CSF were 47 and 25 cm³ respectively; the ventricular volume was fixed at 20 cm³ for the analysis. These are reasonable volumes calculated from the dynamics of methotrexate flux between various CSF compartments; they do not represent specific anatomic compartments. Fourth, a good fit of the observed data could be obtained for values of bulk methotrexate clearance between 19 and 22 cm³/hour, which is consistent with the calculated value of bulk CSF clearance (22 cm³/hour) (7).

[7] Bulk clearance of CSF was assumed to occur at the same rate as CSF production, 22 cm³/hour (7).

[8] Shapiro WR, Young DF, and Mehta BM. Unpublished results.



Compartments	Assumptions
1 Brain	$k_1 - k_2 = k_6$
2 Ventricles	$k_3 - k_4 = k_5$
3 Basal Cistern	$k_7 - k_8 = k_9$
4 Lumbar Sac	k_5 0

$$V_1 dC_1/dt = k_7(C_2 - C_1)$$

$$V_2 dC_2/dt = (k_7 - k_9)C_1 - (k_1 + k_7)C_2 + (k_1 - k_8)C_3$$

$$V_3 dC_3/dt = k_1 C_2 - (k_1 + k_3)C_3 + (k_3)C_4$$

$$V_4 dC_4/dt = k_3(C_3 - C_4)$$

FIGURE 3.—Compartment model of methotrexate flow dynamics in the CSF.

Periventricular Brain Tissue Profiles

The profiles of methotrexate concentration in the extracellular fluid (ECF) of periventricular brain tissue at various times after a single intraventricular administration have been computed and are shown in figure 4. The ventricular CSF concentration of methotrexate for each brain tissue profile is given as the ordinate intercept. The CSF concentrations were falling with time and correspond to the ventricular concentrations measured by Shapiro et al (2) (fig 2). The initial ventricular methotrexate concentration was calculated from the clinically administered dose (6.25 mg/m²) assuming rapid equilibration in a ventricular volume of 20 cm³; a value of 1×10^{-3} M was obtained. The apparent brain tissue diffusion constant of methotrexate was assumed to be the same as that measured in the rhesus monkey, 1.2×10^{-6} cm²/second (1). Diffusional transport was calculated along rectangular coordinates into an infinitely thick slab of brain. Neither capillary exchange nor metabolism of methotrexate (which would effectively lower the ECF concentration of drug) was assumed.

Several observations can be made. The periventricular brain tissue concentration profile of methotrexate changes considerably between 1 and 48 hours. The change is due to the continuous diffusion of methotrexate through the ECF down its concentration gradient as well as to the rapid decrease in CSF levels. There is a 1–2-log decrease in the maximum periventricular methotrexate concentration over 48 hours in comparison to the initial ventricular methotrexate concentration. The decrease in tissue concentration is most rapid during the first several hours after intraventricular administration. The diffusion gradient (tissue concentration profile) into the brain becomes less steep the longer diffusion continues. The longer post-perfusion profiles demonstrate that the deeper brain tissue (1.0–1.5 cm from the ependymal surface) does reach methotrexate levels somewhat $> 10^{-6}$ M or < 3 logs below the initial ventricular concentration. If the therapeutic ECF-tissue concentration of methotrexate is 10^{-6} M, therapeutic concentrations would be achieved in the brain at distances > 1.0 and 1.5 cm from the ependymal surface by 24 and 48 hours respectively.

A final observation is that the ventricular CSF concentration is significantly below the periventricular ECF concentration by 8 hours after intraventricular methotrexate administration. The brain to CSF concentration gradient progressively increases over 48 hours. During this period methotrexate will diffuse from the brain ECF (region of relatively high concentration) into the CSF (region of relatively low concentration) as well as continue to diffuse further into the brain as discussed above. During the initial period after intraventricular methotrexate administration, a certain fraction of the administered dose diffuses into and through brain tissue adjacent to the ventricles and the subarachnoid space. As

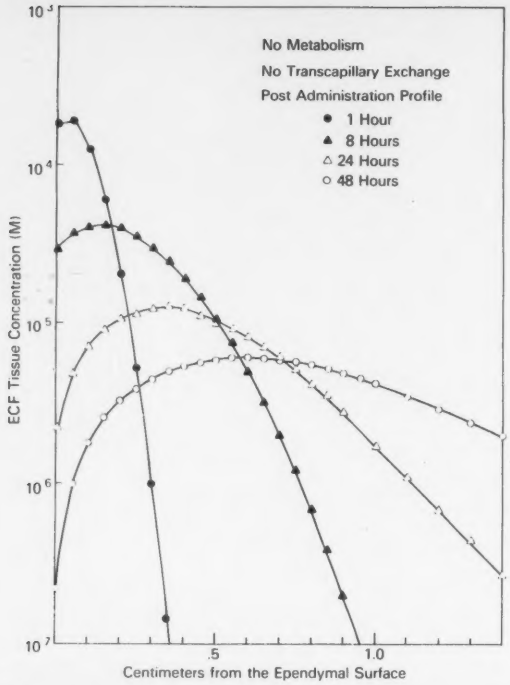


FIGURE 4.—Simulation of periventricular methotrexate concentration in the ECF at various times after intraventricular administration. Diffusional transport was calculated along rectangular coordinates into an infinitely thick slab of tissue. No ECF loss of methotrexate was assumed. The concentration of methotrexate in the ventricular CSF for each concentration profile is given by the ordinate intercept and corresponds to the value measured by Shapiro et al (fig 2) (2).

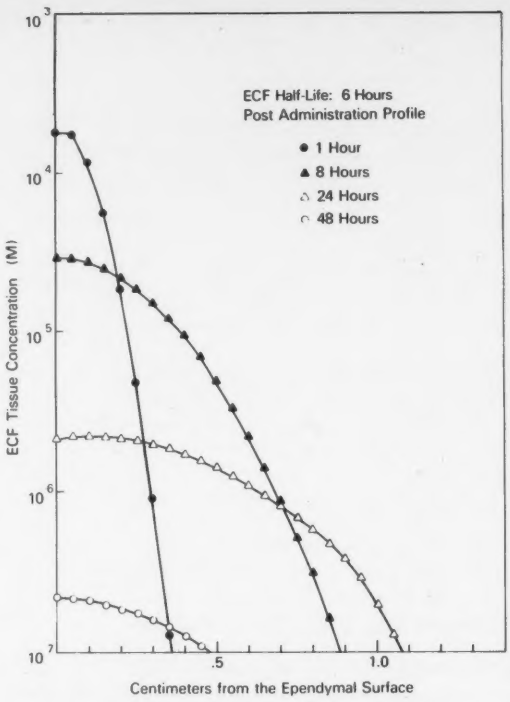


FIGURE 5.—Same as fig 4 except that the ECF half-time (metabolism plus transcapillary loss) was 6 hrs.

methotrexate is cleared from the CSF, the CSF concentration falls and the methotrexate in the surrounding brain tissue will back diffuse into the CSF. Since the ventricular CSF levels have fallen > 1 log below the periventricular ECF levels by 48 hours after intraventricular administration, the back diffusion of tissue methotrexate could have an increasingly significant effect on CSF methotrexate concentration in the post-administration period.

We have attempted to calculate whether there is a significant back flux of methotrexate into the CSF from the brain at 48 hours, when the simulated brain to CSF gradient is very steep. The calculated flux produces a concentration increase in the CSF of $< 10^{-7}$ mmols/cm³/hour.[9] This calculation would suggest that the flux of methotrexate from brain tissue back into the CSF contributes negligibly to ventricular CSF levels given the approximations involved. The persistence of ventricular methotrexate levels is more likely due to the effect of methotrexate reflux from lumbar (spinal) CSF to basal cistern to ventricular CSF (fig 2). This is consistent with the flow constants calculated from the model of methotrexate flow dynamics (fig 3). In further support for reflux of methotrexate into the ventricles is the 6–8-hour ventricular half-time that was observed by Shapiro et al (table 1). The ventricular methotrexate half-time is considerably longer than the ventricular CSF turnover time of 0.9 hours.[10] Methotrexate must be continually re-entering the ventricular CSF; this re-entry is more likely due to reflux from the basal cistern CSF than to reflux from periventricular brain tissue.

[9]CSF concentration change $\doteq D(dC/dx)_i \cdot ECS \cdot$ surface area $\cdot$ 1/exchangeable CSF volume, where $D = 1.2 \times 10^{-6}$ cm²/second; $(dC/dx)_i = < 2 \times 10^{-5}$ mM/cm⁴ (the concentration gradient at the CSF-brain interface); ECS = 0.25; surface area = ≤ 200 cm²; exchangeable CSF volume = ≥ 65 cm³ (ventricles, basal cistern, and peri-basal cistern CSF).

[10]Ventricular CSF turnover should occur at the same rate as CSF production (22 cm³/hr); by assuming a ventricular volume of 20 cm³, a ventricular CSF turnover time of 0.9 hrs may be calculated (7).

Effect of Capillary Permeability and Metabolism

Much of the previous discussion was based on the assumption that methotrexate behaves as an "ideal" drug for intrathecal administration: that is, a drug which does not undergo metabolism (remains in its administered form) and does not permeate brain capillaries (no transcapillary loss). Since drug metabolism and capillary permeability affect the tissue ECF concentration profile in a similar way, these effects are additive. The summed effects of drug metabolism and transcapillary exchange will be expressed as a single term, the ECF half-time of the drug. Let us assume that methotrexate has a brain tissue ECF half-time of 6 hours which would primarily reflect its transcapillary loss from the brain. The resultant post-administration tissue ECS profiles for methotrexate, which were simulated with these new conditions, are shown in figure 5. The ventricular methotrexate levels in the post-administration period were decreasing and are given as the ordinate intercept for each tissue profile. They correspond to the same values as those in figure 4 and were obtained from the study of Shapiro et al (2) (fig 2).

Several comparisons between figures 4 and 5 can be made. The most striking difference is the persistence of a steep diffusion gradient (tissue concentration profile) into the brain in figure 5. This concentration gradient is maintained because methotrexate is continually being removed from the brain ECF, either by metabolism or transcapillary loss. The resultant maximum tissue concentration and tissue concentration in deeper brain tissue (≥ 1.0 cm from the ependymal surface) of methotrexate are significantly less in figure 5 compared to figure 4, particularly for the 24- and 48-hour post-perfusion profiles. A tissue ECF concentration $> 10^{-6}$ M never extends much beyond 0.8 cm from the ependymal surface and by 30 hours all periventricular brain tissue is $< 10^{-6}$ M.

The periventricular ECF concentration profiles of methotrexate in figure 5 suggest that there is little or no back diffusion of methotrexate from brain to CSF since little or no back diffusion gradient exists. This is consistent because the ventricular methotrexate clearance half-time that was calculated from the data of Shapiro et al (table 1) is approximately the same as the brain tissue ECF half-time of methotrexate that was used for the post-perfusion tissue profiles in figure 5, a value of 6 hours.

The graphic simulations clearly demonstrate that very small amounts of drug penetrate the brain from the CSF when the ECF half-time is short, ie, ≤ 6 hours. A convenient rule of thumb is that the tissue ECF concentration profile will approach its maximum penetration of the brain by four or five ECF half-times. For an ECF half-time of 6 hours, the 24-hour post-administration profile approaches the maximum penetration of the brain.

Effect of Two Opposing CSF Surfaces

The previous discussions and the graphic simulations of brain ECF methotrexate concentration have assumed rectangular diffusion coordinates through an infinitely thick slab of brain tissue. Clearly, the human brain has finite thickness. The complex surface anatomy of the brain including sulci, cisterns, and ventricles offers a distinct advantage from the viewpoint of drug delivery from CSF to brain tissue by diffusion. Diffusion distances are reduced and the total CSF-brain surface area is increased. The distance between one CSF surface and another for most human brain tissue is 1.0–1.5 cm; the greatest distance is through the basal ganglia which is about 3.0–3.5 cm.[11] Therefore, the maximum diffusion distance from a CSF surface for most brain tissue is 0.5–0.8 cm and the longest diffusion distance through the basal ganglia is 1.5–1.8 cm.

Assuming there is reasonably good equilibration of drug throughout the CSF, so that the concentration of drug at the opposing CSF surfaces of the brain is approximately the same, the actual post-administration brain ECF concentration profiles of drug will be hyperbolic from one CSF-brain surface to the opposing CSF-brain surface. Drug will diffuse across both CSF-brain interfaces into and through the brain ECF; diffusion will proceed down their respective concentration gradients toward the center of the tissue. This condition has been simulated for two brain thicknesses. The brain ECF concentration profiles of four graphs are shown in figure 6. Again, the model drug is methotrexate with an apparent brain diffusion constant of 1.2×10^{-6} cm^2/second. The left two graphs illustrate a brain thickness of 1.0 cm; the right two graphs illustrate a brain thickness of 3.0 cm. The upper two graphs illustrate the condition with no loss of methotrexate from the ECF (no metabolism and no transcapillary loss); the lower two graphs illustrate the effects of an ECF half-time of 6 hours (the summed effects of metabolism and transcapillary loss). Again rectangular diffusion coordinates are assumed. The CSF concentration of methotrexate at opposing brain surfaces is assumed to be equal for each graph in

[11] The brain tissue thickness between opposing CSF-brain surfaces was obtained from multiple measurements of 1-cm coronal sections from 10 consecutive adult human autopsy brains previously fixed in 10% formalin for 1–3 wks.

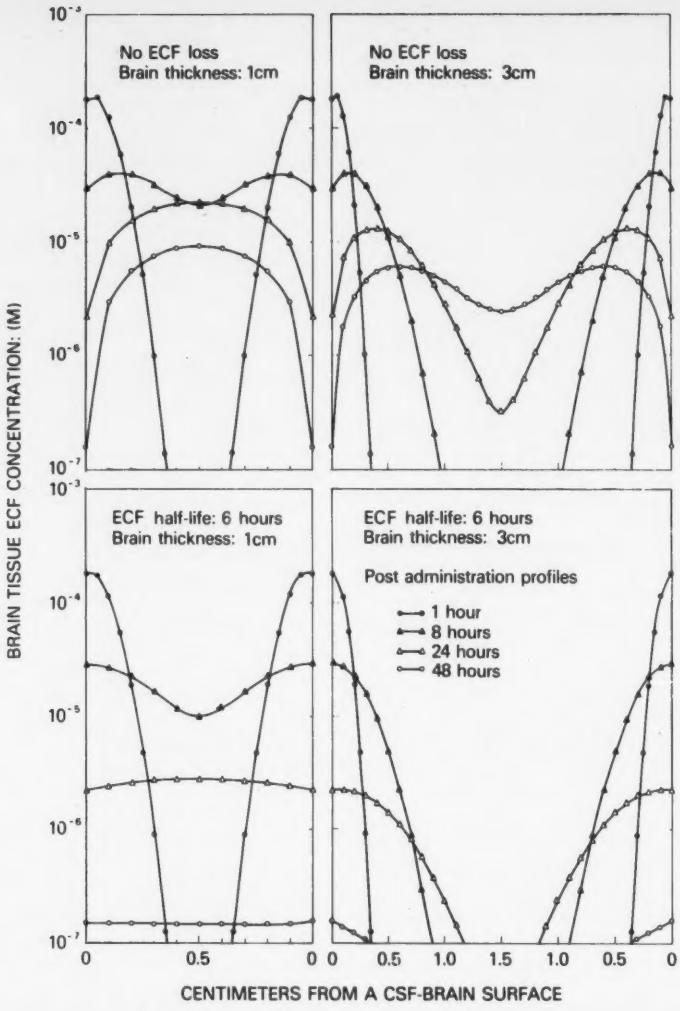


FIGURE 6.—Composite of 4 graphs consistent with fig 4 except for the following: (*a*) diffusional transport is calculated from 2 opposing surfaces (1 cm apart in the left 2 graphs and 3 cm apart in the right 2 graphs); (*b*) the upper 2 graphs assume no ECF loss of methotrexate; and (*c*) the ECF half-time was 6 hrs in the lower 2 graphs.

figure 6 and is given as the ordinate intercept.[12] The CSF concentration for each tissue profile corresponds to the ventricular methotrexate concentration measured by Shapiro et al (2) and is identical to figures 4 and 5. Therefore, figures 4–6 are comparable.

[12]Similar profiles could be constructed for conditions in which the CSF concentration was not equal at the opposing CSF-brain surfaces or for conditions in which the CSF concentration was changing at different rates at the opposing CSF-brain surfaces.

For discussion purposes we can consider the graphs in figure 6 to represent two neighboring gyri, one 1-cm thick and the other 3-cm thick. Without repeating the discussion of the previous sections (which is also applicable to fig 6) several additional points may be made. Significantly higher concentrations of methotrexate are reached in the center of the smaller 1-cm-thick tissue (or gyrus) in comparison to the larger 3-cm-thick brain. The concentration at the center will reflect the additive effects of

diffusional transport from both CSF-brain surfaces. Again, the effect of a short ECF half-time of methotrexate (6 hours) is shown. It is clear that very little drug reaches the center of the 3-cm-thick brain (or gyrus) by diffusional transport from the CSF-brain surfaces; the calculated values at 24 and 48 hours are 2.4 and 1.4 $\times$ 10^{-8} M respectively.

Multiple-Dose Scheduling

Repetitive or multiple-dose scheduling could significantly increase the concentration of methotrexate in periventricular brain tissue. If methotrexate was re-administered intraventricularly 24 hours after the initial dose, the tissue concentration profile of methotrexate at 25 hours would be approximately the sum of the 1- and 24-hour profiles in figures 4–6. Similarly, the CSF methotrexate concentration would be expected to be approximately the sum of the 1- and 24-hour concentrations 1 hour following the second administration. If the drug was administered on 3 consecutive days, the tissue concentration profile 1 hour after the last dose would be approximately the sum of the 1-, 24-, and 48-hour profiles in the same figures. Similarly, the CSF methotrexate concentration would be expected to be approximately the sum of the respective 1-, 24-, and 48-hour concentrations. Therefore, multiple-dose scheduling can significantly increase both the CSF and the brain tissue concentration of methotrexate as well as increase the duration that the concentration is maintained.

Conclusions

The primary limiting factors of drug delivery to brain tissue after intrathecal drug administration are (*a*) drug distribution between the CSF compartments; (*b*) drug CSF clearance; (*c*) apparent diffusion constant of the drug through the brain ECF and the distance between opposing CSF surfaces; (*d*) brain ECF half-time of the drug (due to drug metabolism and drug loss across brain capillaries); and (*e*) CNS and systemic toxicity of the drug.

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Extraction and Analytic Procedures for Cytosine Arabinoside and 1-β-D-Arabinofuranosyluracil and Their 5′-Mono-, Di-, and Tri-phosphates[1]

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SUMMARY

A qualitative and quantitative comparison of perchloric acid (PCA) and trichloroacetic acid (TCA) extraction procedures of biologic material containing cytosine arabinoside (Ara-C) and 1-β-D-arabinofuranosyluracil (Ara-U) and their biologic 5′-phosphate derivatives revealed definite superiority of the PCA procedure over the TCA procedure. High-pressure liquid chromatography provides rapid and accurate quantitative and qualitative separation of both Ara-C and Ara-U and their 5′-mono-, di-, and tri-phosphates (μBondapak NH_2 column), or their 5′-mono-, di-, and tri-phosphates without nucleosides (ABX Permaphase columns).

[Cancer Treat Rep 61:643–646, 1977]

The quantitative and qualitative analysis of pyrimidine nucleosides and nucleotides has been accomplished in the past by paper, thin-layer, and column chromatography (1–3). Extraction of nucleosides and nucleotides from cell homogenates or enzyme assays was normally done with perchloric acid (PCA) or trichloroacetic acid (TCA) (1–3), most often according to the procedure of Schmidt-Tannhäuser (4).

In order to evaluate these techniques qualitatively and quantitatively, P815-bearing mice were injected with tritiated cytosine arabinoside (^{3}H-Ara-C) and the ascites were submitted to different extraction and filtration methods.

A comparison of the efficiency of these procedures, including a filtration procedure, and extensive studies on the separation of Ara-C-derived nucleotides and nucleosides by high-pressure liquid chromatography (HPLC) are presented. The recommended extraction procedure, together with the HPLC separation, should be a valuable tool in the analysis of metabolic products of Ara-C and other pyrimidine and probably purine analogs.

MATERIALS AND METHODS

Ten to 20 BDF_1 mice were inoculated ip with about 10^6 P815 cells; ascites-bearing mice (5 or 6 days after inoculation) were injected with 20 mg/kg of ^{3}H-Ara-C (specific activity, 2.22 millicuries/mmol) and the total ascitic fluid was harvested 1 hour after the injection. The ascites cells were then sedimented by centrifugation, submitted to a hypotonic shock for the elimination of erythrocytes, and further washed with 0.25 M sucrose. Aliquots of the cell suspension in 0.25 M sucrose were submitted to homogenization in a Potter-glass-teflon homogenizer. The homogenate was divided into three equal volumes for subsequent extraction with TCA or PCA or filtration through a 43-mm Diaflo Amicon PM-30 filter (Amicon Corp, Lexington, Mass). The TCA and PCA extractions were done according to the Schmidt-Tannhäuser technique (4) which consisted of adding 2.5 ml of 0.33 M PCA or 10% TCA per 1 ml of homogenate. The precipitate was washed twice with 1.0 ml of the same strength of PCA and TCA. The PCA supernatant was neutralized immediately to pH 7.0 with 2 N KOH. Potassium perchlorate was eliminated by centrifugation after storage at 4°C for at least 2 hours. The resulting supernatant was adjusted to pH 4.5 with 1 N formic acid.

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[3]We are indebted to Dr. Irwin H. Krakoff for his continuous interest.

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The TCA supernatant was adjusted immediately to pH 4.5 with 2 N ammonium bicarbonate. Aliquots (up to 14 ml) of the two extracts were then applied to an AG1-X8, 200–400 mesh (Bio-Rad, Richmond, Calif) column (200 × 8 mm) and eluted subsequently with water, 0.005 N HCl, 0.1 N HCl, 0.5 N HCl (HCl elution procedure) or water, 1 N ammonium bicarbonate, 2 N ammonium bicarbonate (bicarbonate elution procedure).

Each eluate was lyophilized either at room temperature for 4, 6, or 16 hours or cooled to −40°C and lyophilized for 6 hours.

For the Amicon filtration (Amicon filtration procedure), 1 ml of the crude homogenate was diluted with 24 ml of water and filtered through the PM-30 filter. The remaining material was washed with 25 ml of 10%, 50%, and 100% ethanol and with 0.1 and 0.2 N HCl.

For the qualitative analysis of the fractions in the elution and filtration procedure, paper chromatography, as described earlier, was used (2). For the quantitative evaluation of the respective fractions, radioactivity was evaluated in a Packard Tricarb liquid scintillation spectrometer, Model 3380 (Packard Instrument Corp, Downers Grove, Ill) either by direct counting in Diotol (5) or after combustion in a Packard sample oxidizer, Model 306 (Packard Instrument Corp, Downers Grove, Ill).

For the separation of nucleotides derived from Ara-C and 1-β-D-arabinofuranosyluracil (Ara-U), a mixture of the six nucleotides was injected into two serially connected ABX-permaphase (DuPont, Wilmington, Del) columns (2.7 mm × 60 cm). A Waters (Milford, Mass) high-pressure liquid chromatography apparatus, Model 202 with Waters U6K injector, was the instrument used. The elution, performed at ambient temperature, was done within 10 minutes with a linear gradient from 0.005 to 0.2 M potassium phosphate monobasic buffer (Fisher, Pittsburgh, Pa), pH 3.0, at a pressure of 3000–3500 psi. The flow rate was 3.0 ml/minute, and an ultraviolet (UV) detector at wavelength 254 nm was used.

For the simultaneous separation of nucleosides and nucleotides of Ara-C and Ara-U in 3–10 μl, a 0.64 × 30 cm μBondapak NH_2 (Waters, Milford, Mass) column was used. A linear gradient from 0.002 to 0.4 M monobasic potassium phosphate buffer (Fisher Scientific Co, Fair Lawn, NJ), pH 2.1 (initial) to pH 5.0 (end), at ambient temperature and at a pressure of about 1500 psi was employed as the eluant. The flow rate was 3.0 ml/minute and the chart speed was 0.75 inch/minute; a UV detector at wavelength 254 nm, at 0.16 OD unit full scale, was used. 5′-Mono-, di-, and tri-phosphates of Ara-C and Ara-U were purchased from Terra-Marine Bioresearch (La Jolla, Calif).

RESULTS AND DISCUSSION

About 1% of the radioactivity injected ip into the tumor-bearing mice was taken up by the ascites cells. When the crude PCA and TCA extracts were submitted to paper chromatography, it was found (table 1) that the PCA extracts contained considerably higher amounts of di- and tri-phosphates than did the TCA extracts. The increased amounts of mono-phosphates in the TCA extract suggest a breakdown of di- and tri-phosphates. Factors accelerating the breakdown of di- and tri-phosphates are prolonged extraction time, elevated temperatures, delayed neutralization, and freezing and thawing of the extracts. In an earlier report of the extraction of solid P815 tumors (2), we noted the breakdown of di- and tri-phosphates only after prolonged freezing of the TCA extracts. It is also possible that tissue-specific enzymes accelerate the breakdown during removal and homogenization of the tumor. We suggest that preliminary tests for the evaluation of this phenomenon be performed in each individual case. According to Brown and Miech (6), TCA extraction of adenosine tri-phosphates produces di- and mono-phosphates only when the extracts are not immediately neutralized. TCA extraction procedures have been used widely for the extraction and HPLC analysis of nucleotides from biologic samples (7,8). When TCA is used in in vitro reactions for the precipitation of protein, the final concentration of TCA is considerably lower and the breakdown of 1-β-D-arabinofuranosylcytosine-5′-triphosphate (Ara-CTP) has not been noticed under those conditions.

Filtration of the crude cell homogenate through a PM-30 Amicon filter provides good recoveries of the radioactivity in the filtrate. However, extensive breakdown of di- and tri-phosphates occurred during the filtration process, and this method was not pursued any further.

The HPLC of the extracts can be performed either directly on a μBondapak NH_2 column or on an ABX Permaphase column after separation of nucleosides and nucleotides on an AG1-X8 ion exchanger. Only nucleotides can be separated on ABX Permaphase. Since ABX Permaphase gives somewhat better re-

TABLE 1.—Paper chromatographic analysis (ref 2) of crude PCA and TCA extracts*

Extract	Di- + tri-phosphates of Ara-C	Mono-phosphates of Ara-C	Ara-C	Ara-U
PCA	66	3	13	6
TCA	38	27	23	1

*Average of 2 experiments expressed in % of total counts on paper chromatogram.

TABLE 2.—Recovery of radioactivity after column chromatography of PCA and TCA extraction in % of applied radioactivity

Extract	Eluant	Average of experiments 1 and 2 (% radioactivity)
PCA	H_2O + 0.005 N HCl	10.3
PCA	0.1 + 0.5 N HCl	84.1
Total		94.4
PCA	H_2O	9.6
PCA	1.0 + 2.0 N ABC*	89.5
Total		99.1
TCA	H_2O + 0.005 N HCl	40.9
TCA	0.1 + 0.5 N HCl	56.4
Total		97.3
TCA	H_2O	43.2
TCA	1.0 + 2.0 N ABC*	60.5
Total		103.7

*ABC = ammonium bicarbonate.

sults, the preparative steps for the Permaphase columns have been evaluated qualitatively and quantitatively (9). Both PCA and neutralized TCA extracts are adjusted to pH 4.5 just prior to the chromatography on an AG1-X8 ion exchanger. The recovery, as indicated in table 2, reveals significant differences in the water washes (in the elution with HCl the water wash also includes a wash with 0.005 N HCl). While the two washings (consisting theoretically of nucleosides only) in the PCA extraction procedure contained only about 10% of the total counts, the corresponding fractions in the TCA extraction procedure contained about 42% of the total counts, indicating a discrepancy in the quantitative aspect of the two extraction procedures. Consequently, the elution of the nucleotides (with 0.1 + 0.5 N HCl or 1.0 + 2.0 N ammonium bicarbonate) also revealed differences, whereas the total recovery from the column was comparable for all four chromatographic procedures. Analysis for the qualitative results (table 3) gives satisfactory separation of nucleosides and nucleotides for the PCA but not for the TCA extraction procedure. Obviously, minor overlap of nucleosides into the nucleotide eluant and vice versa did take place also in the PCA extraction procedure. In this procedure, the elution with 1 N ammonium bicarbonate may be slightly more efficient in separating nucleosides from nucleotides than is the elution with 0.1 N HCl.

Much more obvious is the insufficient separation of nucleosides and nucleotides in the TCA extraction procedure. The capacity of the column was large enough to assure adequate handling of the large amounts of TCA.

The eluates from the AG1-X8 column were concentrated by high vacuum lyophilization. No breakdown of Ara-C di- and tri-phosphates was noticed in either the 0.1 N HCl or the 1.0 N ammonium bicarbonate eluates derived from the PCA extraction procedure during any of the four different lyophilization procedures. In the TCA extraction procedure, the 0.1 N HCl eluate exhibited no breakdown during lyophilization overnight; the ammonium bicarbonate eluate, however, clearly demonstrated breakdown of the di- and tri-phosphates; neutralization of the eluates before lyophilization had no beneficial effect upon the stability of the di- and tri-phosphates. The completely dried nucleotides were then redissolved in water and were ready for the separation on the ABX Permaphase columns. An example for the separation of the six Ara-C- and Ara-U-derived nucleotides on ABX Permaphase is reported elsewhere (9). While the separa-

TABLE 3.—Paper chromatographic analysis of PCA and TCA extracts for nucleoside and nucleotide separation on AG1-X8 column*

Extract	Eluant	Nucleosides (%)	Nucleotides (%)	Total
PCA	H_2O†	83	8	91
	0.1 N HCl‡	11	77	88
PCA	H_2O†	72	4	76
	1.0 N ABC†,§	5	81	86
TCA	H_2O	49	39	88
	1.0 N HCl†,‡	0	82	88
TCA	H_2O	46	43	89
	1.0 N ABC†,§	0	88	88

*As evaluated according to ref 2 for each eluant.
†Does not include the 0.001 N eluant.
‡Does not include the 0.5 N HCl eluant.
§Does not include the 2 N ABC eluant.

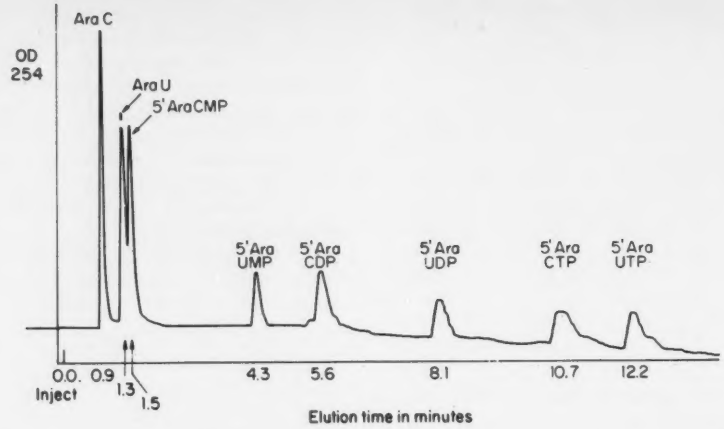

FIGURE 1.—Separation of aqueous solution of Ara-C and Ara-U and their 5'-mono-, di-, and tri-phosphates by HPLC on μBondapak NH_2. Individual nucleosides and nucleotides are marked on top of their respective peaks. (For method, *see* Materials and Methods.)

tion of nucleosides and nucleotides is a prerequisite for the use of the Permaphase columns, the μBondapak NH_2 column allows the separation of mixtures of nucleosides and nucleotides (fig 1).

It is essential that the volumes injected into the HPLC columns are kept as small as possible, ie, 5 μl is well tolerated in our hands.

The use of radioactive ^{3}H-Ara-C for biologic experiments allows accurate evaluation of as little as 100 pmols of any of the two nucleosides and six potential nucleotide metabolites.

Ara-CTP remains unchanged during the HPLC on both columns.

This procedure will allow the establishment of metabolic patterns in biologic material. Adaptation of this method to other pyrimidine and probably purine nucleosides and nucleotides should be possible.

LIST OF COMPOUNDS

Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Uracil arabinoside: NSC-68928; CAS reg. No. 3083–77–0; uracil, 1-β-D-arabinofuranosyl-
1-β-D-Arabinofuranosylcytosine-5-diphosphate: Ara-CDP
1-β-D-Arabinofuranosylcytosine-5'-monophosphate: Ara-CMP
1-β-D-Arabinofuranosylcytosine-5'-triphosphate: Ara-CTP
1-β-D-Arabinofuranosyluracil-5'-diphosphate: Ara-UDP
1-β-D-Arabinofuranosyluracil-5'-monophosphate: Ara-UMP
1-β-D-Arabinofuranosyluracil-5'-triphosphate: Ara-UTP

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Aspects of the Reversal of Methotrexate Toxicity in Rodents [1,2]

R. L. Kisliuk,[3,*] M. H. N. Tattersall,[4,5] Y. Gaumont,[3] E. J. Pastore,[6] and B. Brown[4]

SUMMARY

An ip injection of a *Lactobacillus casei* dihydrofolate reductase preparation into rats and mice given a single lethal dose of methotrexate (MTX) caused a marked lowering of free MTX in the blood. Alternatives to citrovorum factor as agents for reversing MTX toxicity were explored in mice. *dl*,L-5-Methyltetrahydrofolate, *dl*,L-5,10-methylenetetrahydrofolate, *l*,L-5,10-methylenetetrahydrofolate, and dihydrofolate were also effective MTX antagonists; *d*,L-5,10-methylenetetrahydrofolate was inert.

[Cancer Treat Rep 61:647–650, 1977]

The present experiments were designed and carried out in response to a request from Dr. Edward J. Modest of the Sidney Farber Cancer Center for new approaches to alleviate toxicity encountered after high-dose methotrexate (MTX) therapy.

METHODS

MTX was assayed utilizing *Lactobacillus casei* (*L. casei*) dihydrofolate reductase as described previously (1). The vehicle for all reduced folates was 2% $NaHCO_3$ and 1% Na ascorbate. *dl*,L-Tetrahydrofolate[7] was purchased from Sigma Chemical Co, St. Louis, Mo, and assayed (2) by its activity for *L. casei* thymidylate synthetase. *dl*,L-5,10-Methylenetetrahydrofolate was prepared by mixing formaldehyde and *dl*,L-tetrahydrofolate in a ratio of 2:1(3). Diastereoisomers of *dl*,L-5,10-methylenetetrahydrofolate were separated by DEAE chromatography (4). Dihydrofolate was synthesized by dithionite reduction (5). *dl*,L-5-Methyltetrahydrofolate was purchased from Terra-Marine, La Jolla, Calif, and its authenticity verified by its ability to support the growth of *L. casei* (ATCC-7469) but not *Streptococcus faecium* var. *durans* (ATCC-8043). The rats used were Charles River CD males (100–125 g) and the mice used were BDF_1 males (22–25 g). Crude (2% pure) *L. casei* dihydrofolate reductase was obtained by lyophilizing active fractions from a phosphocellulose column used to purify thymidylate synthetase (6). Prior to injection the material was dissolved in water and dialyzed for 12 hours at 3°C against normal saline. Pure dihydrofolate reductase was obtained by affinity chromatography on pteroyllysine sepharose (7) eluted with 0.5 M KCl and 50 mM potassium phosphate (pH 8.0). Both dihydrofolate reductase preparations were free of folates as assayed by *L. casei*. In addition, folates could not be detected spectrophotometrically in the pure preparation. One mol of NADPH (Sigma) was added per mol of dihydrofolate reductase to enhance its affinity for MTX (8). *L. casei* was grown at the New England Enzyme Center (9).

RESULTS AND DISCUSSION

As shown in figure 1, administration of dihydrofolate reductase ip to rats or mice caused a lowering of the free blood MTX levels presumably by binding the MTX to the enzyme. The two rats that received

[1] Supported by Public Health Service grants CA-06516 (Drs. Tattersall and Brown) and CA-10914 (Drs. Kisliuk and Gaumont) from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare; and grant GB-41533 (Dr. Pastore) from the National Science Foundation.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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[4] Division of Medical Oncology, Sidney Farber Cancer Center, Harvard Medical School, Boston, Mass.

[5] *Present address.* Ludwig Institute for Cancer Research, Sydney, Australia.

[6] Department of Chemistry, University of California, San Diego.

[7] *d* and *l* refer to the unnatural and natural configurations of carbon 6 respectively.

[*] *Reprint requests to:* Dr. R. L. Kisliuk, Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Mass 02111.

TABLE 1.—Effect of pure dihydrofolate reductase on the survival of mice treated with MTX

Experiments	Drug	Dose	Route	Schedule	No. of deaths/ No. of mice	Day of death
a	None	—	—	—	0/10	—
b	MTX	450 mg/kg	ip	qd × 1	6/10	5,5,5,5,5,6
c	MTX	450 mg/kg	ip	qd × 1	5/10	6,6,6,7,9
	Dihydrofolate reductase	1 mg/mouse	ip	qd × 1, 6 hrs after MTX		
d	MTX	450 mg/kg	ip	qd × 1	8/10	5,6,6,6,6,6,6,7
	Dihydrofolate reductase	1 mg/mouse		qd × 1, 24 hrs after MTX		
e	MTX	450 mg/kg	ip	qd × 1	0/10	—
	Citrovorum factor	200 mg/kg	ip	qd × 1, 6 hrs after MTX		
f	MTX	450 mg/kg	ip	qd × 1	0/10	—
	Citrovorum factor	200 mg/kg	ip	qd × 1, 24 hrs after MTX		

crude enzyme in this experiment survived whereas all four controls died within 2 weeks. The mice treated with pure enzyme 6 hours after MTX showed a hint of increased survival (table 1). Those treated 24 hours after MTX showed no increased survival.

Assuming a total fluid volume of 100 ml/rat and a concentration of free MTX of 10^{-6} M, it would take 1.8 mg of dihydrofolate reductase (molecular weight, 18,000) to bind all of the free MTX. A total of 5 mg of enzyme was administered to each rat in a total volume of 1.7 ml containing 250 mg of protein. Assuming a total fluid volume of 20 ml/mouse and a concentration of free MTX of 10^{-6} M, it would take 0.36 mg of enzyme to bind all of the free MTX. A total of 1.0 mg of enzyme was given to each mouse in a total volume of 0.1 ml.

In table 2 it is shown that reduced folates, in addition to citrovorum factor,* are capable of preventing MTX toxicity in mice. *dl*,L-5,10-Methylenetetrahydrofolate, *l*,L-5,10-methylenetetrahydrofolate, *dl*,L-5-methyltetrahydrofolate, and dihydrofolate all showed activity. Similar results were obtained earlier (10) with *dl*,L-tetrahydrofolate, *l*,L-5-methyltetrahydrofolate, and dihydrofolate in reversing the toxicity and antileukemic effect of MTX in mice. In another study (11), *dl*,L-5-methyltetrahydrofolate proved comparable to citrovorum factor in reversing MTX toxicity in mice. The results reported here (table 2, experiments 1e and 2d) show, in addition, that *d*,L-5,10-methylenetetrahydrofolate is inert. Instances are known where *d*,L-derivatives of tetrahydrofolate are inhibitors (12–14). It is to be emphasized that citrovorum factor is a mixture of diastereoisomers which should be taken into account in pharmacologic studies but is usually ignored.

If, as is generally assumed, the lack of the substrate for thymidylate synthetase, *l*,L-5,10-methylenetetrahydrofolate, is crucial to MTX toxicity, it seems reasonable to provide that substance directly rather than the formyltetrahydrofolate derivative with which the formyl group must be removed or reduced prior to its utilization as a substrate.

In view of the complicated metabolism of folates (15) and the possible differences in pharmacokinetics of various derivatives, it would be advisable to prepare the natural diastereoisomers (*l*,L) of citrovorum factor, 5,10-methylenetetrahydrofolate, and 5-methyltetrahydrofolate, and test them further alone and in combination for their ability to antagonize MTX.

*Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

TABLE 2.—Ability of reduced folate derivatives to prevent MTX toxicity in mice*

Experiment	Drug	Dose (mg/kg)	Vehicle	Route	No. of deaths/ No. of mice	Day of death
1a	None	—	—	—	0/8	—
1b	MTX	350	Saline	ip	5/10	5,6,6,6,6
1c	MTX	350	Saline + ascorbate-bicarbonate, 0.25 ml/mouse	ip	4/5	6,6,6,6
1d	MTX + *dl*,L-5,10-methylenetetrahydrofolate	350	Saline	ip		
		1	Ascorbate-bicarbonate	sc	1/5	5
		2	Ascorbate-bicarbonate	sc	1/5	6
		5	Ascorbate-bicarbonate	sc	2/5	5,6
		10	Ascorbate-bicarbonate	sc	2/5	6,8
1e	MTX + *d*,L-5,10-methylenetetrahydrofolate	350	Saline	ip		
		1	Ascorbate-bicarbonate	sc	3/5	6,6,6
		2	Ascorbate-bicarbonate	sc	1/5	6
		5	Ascorbate-bicarbonate	sc	4/5	6,6,6,7
		10	Ascorbate-bicarbonate	sc	2/5	5,7
1f	MTX + *l*,L-5,10-methylenetetrahydrofolate	350	Saline	ip		
		1	Ascorbate-bicarbonate	sc	2/5	6,10
		2	Ascorbate-bicarbonate	sc	0/5	—
		5	Ascorbate-bicarbonate	sc	0/5	—
		10	Ascorbate-bicarbonate	sc	0/5	—
1g	MTX + dihydrofolate	350	Saline	ip		
		1	Ascorbate-bicarbonate	sc	1/5	6
		2	Ascorbate-bicarbonate	sc	3/5	6,7,7
		5	Ascorbate-bicarbonate	sc	1/5	6
		10	Ascorbate-bicarbonate	sc	1/5	6
1h	MTX + citrovorum factor	350	Saline	ip		
		1	Ascorbate-bicarbonate	sc	1/5	7
		2	Ascorbate-bicarbonate	sc	4/5	6,6,6,6
		5	Ascorbate-bicarbonate	sc	1/5	6
		10	Ascorbate-bicarbonate	sc	1/5	6
2a	None	—	—	—	0/10	—
2b	MTX	450	Saline	ip	10/10	5,5,5,6,6,6,6,7,13
2c	MTX + *dl*-L-5,10-methylenetetrahydrofolate	450	Saline	ip		
		1.25	Ascorbate-bicarbonate	sc	4/5	5,13,14,14
		2.5	Ascorbate-bicarbonate	sc	2/5	5,13
		5	Ascorbate-bicarbonate	sc	4/5	6,6,6,15
		10	Ascorbate-bicarbonate	sc	1/5	4
2d	MTX + *d*,L-5,10-methylenetetrahydrofolate	450	Saline	ip		
		1.25	Ascorbate-bicarbonate	sc	3/5	5,6,7
		2.5	Ascorbate-bicarbonate	sc	5/5	5,5,5,7,8
		5	Ascorbate-bicarbonate	sc	5/5	4,5,5,5,6
		10	Ascorbate-bicarbonate	sc	5/5	5,5,5,5,6
2e	MTX + *dl*,L-5-methyltetrahydrofolate	450	Saline	ip		
		1.25	Ascorbate-bicarbonate	sc	0/5	—
		2.5	Ascorbate-bicarbonate	sc	4/5	6,6,7,8
		5	Ascorbate-bicarbonate	sc	1/5	6
		10	Ascorbate-bicarbonate	sc	0/5	—
2f	MTX + citrovorum factor	450	Saline	ip		
		1.25	Ascorbate-bicarbonate	sc	4/5	6,6,7,8
		2.5	Ascorbate-bicarbonate	sc	1/5	7
		5	Ascorbate-bicarbonate	sc	0/5	—
		10	Ascorbate-bicarbonate	sc	1/5	6

*All drugs were given qd × 1. MTX and folate derivatives were given at the same time.

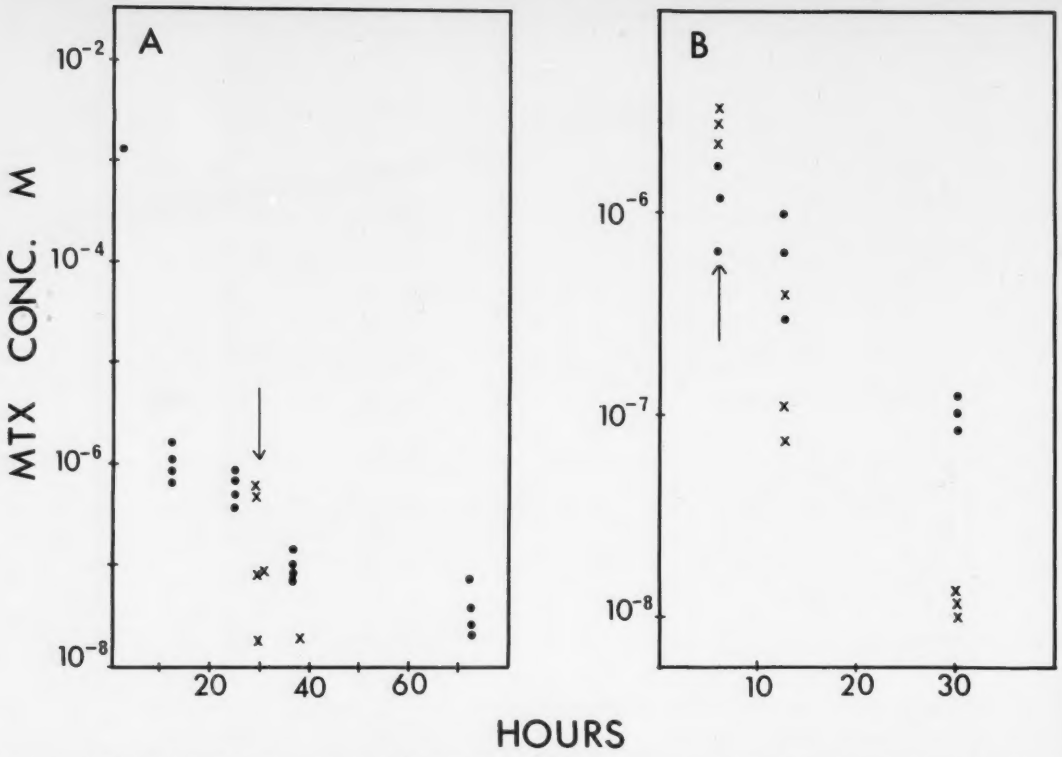

FIGURE 1.—Free blood MTX levels in control and dihydrofolate reductase-treated animals. *A*. (●) = 4 control rats that received 250 mg/kg of MTX ip at time 0. (×) = 2 rats treated with the same dose of MTX. Crude dihydrofolate reductase was given ip at the arrow. The 2 MTX levels at the arrow were obtained immediately prior to injection of dihydrofolate reductase. *B*. (●) = 3 control mice that received 450 mg/kg of MTX ip at time 0. (×) = 3 additional mice treated with the same dose of MTX; pure dihydrofolate reductase was then administered ip at 6 hrs (table 1, experiment C). The 6-hr MTX samples were obtained immediately prior to the injection of dihydrofolate reductase.

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Dose-Dependent Metabolism of Methotrexate in Man and Rhesus Monkeys [1]

Samuel A. Jacobs,* Ronald G. Stoller, Bruce A. Chabner, and David G. Johns [2]

SUMMARY

Humans and rhesus monkeys receiving high-dose methotrexate (MTX) (> 50 mg/kg) excrete significant quantities of the metabolite, 7-hydroxy-MTX. This metabolite, though 200-fold less potent than MTX as an inhibitor of mammalian dihydrofolate reductase, is of very limited aqueous solubility, and thus may contribute to the renal toxicity of the high-dose regimen. The metabolite was not observed in previous pharmacologic studies in which conventional doses of MTX were administered (< 10 mg/kg).

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For > 2 decades the folate antagonist, methotrexate (MTX), has been used extensively in the treatment of neoplastic diseases (1). A wide range of dose schedules have been used in an effort to take advantage of possible differences in biochemical and kinetic growth characteristics of normal and tumor tissues (2). Recently, high-dose MTX (> 50 mg/kg) with citrovorum factor[3] (CF) rescue has added a new therapeutic alternative to the treatment of osteogenic sarcoma (3,4). However, this regimen has been associated with a significant incidence of renal failure (3,5) in addition to the toxicities of bone marrow depression and gastrointestinal ulceration that are seen with conventional MTX therapy. Pharmacologic studies in man and other primates at MTX dose levels of < 10 mg/kg indicate that the drug does not undergo significant metabolic alteration (6–8). A similar refractoriness is seen in most other mammalian species, with the exception of the rabbit and guinea pig, in which biotransformation occurs even at low dose levels (9). In recent work from our laboratory, however, quantitatively significant conversion to the metabolite, 7-hydroxy-MTX, has been demonstrated in man and rhesus monkeys receiving high-dose MTX (> 50 mg/kg) (10). This metabolite, though a much weaker inhibitor of mammalian dihydrofolate reductase than the parent compound, is of extremely limited aqueous solubility, and in this regard may be of clinical significance with respect to the renal toxicity of the high-dose regimen. The evidence, to be described below, suggests that in primates a hepatic MTX oxidase having low substrate affinity catalyzes the conversion of MTX to 7-hydroxy-MTX and accounts for the dose-dependent generation of this metabolite.

METHODS

Five patients with advanced neoplastic disease (11–48 years of age) were treated with high-dose MTX (50–200 mg/kg) by 6-hour iv infusion followed by CF rescue. Three female rhesus monkeys were given either MTX or 3′,5′-7,9(n)^{3}H-MTX (specific activity, 148 microcuries/mmol) at 200 mg/kg by 6-hour iv infusion. Urine was serially collected at 0–6, 6–12, 12–18, and 18–24 hours from the start of the infusion for the patients and at 0–6, 6–12, and 12–24 hours for the monkeys. Urine from each collection period was assayed for dihydrofolate reductase inhibitory activity (11) and ^{3}H-radioactivity by liquid scintillation counting where appropriate. Aliquots of urine, adjusted to pH 8.3, were chromatographed on diethylaminoethanyl (DEAE)-cellulose columns (1.3 × 16 cm) and gradient elution was carried out with ammonium bicarbonate over a concentration range of 0.1–0.4 M with 500 ml in each reservoir (12). One hundred 6-ml fractions were collected, absorbance

[1] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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[3] Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

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TABLE 1.—Urinary excretion of 7-hydroxy-MTX by patients 6–12 and 18–24 hrs after iv administration of MTX (≥ 50 mg/kg)

Patient No.	Dose (mg/kg)	Collection period			
		6–12 hrs		18–24 hrs	
		mg/ml	%*	mg/ml	%
1	200	0.05	2.1	0.16	33
2	100	0.02	4.0	0.04	30
3	100	†	9.6	0.02	22
4	50	0.02	0.7	0.03	7
5	50	0.01	0.9	< 0.01	10

*% of drug excreted as metabolite.
†Incomplete urine collection.

at 300 nm was determined, and in experiments with ^{3}H-MTX, 0.02-ml samples were assayed for ^{3}H-radioactivity. The elution of MTX (fractions 22–28) was determined by dihydrofolate reductase inhibitory activity, ultraviolet and visible absorption spectra, and liquid scintillation counting.

The monkeys were killed 24 hours after the start of the infusion and kidney tissue was prepared for histologic and autoradiographic studies. A portion of the kidney tissue was stored at –8°C for determination of MTX content by enzyme inhibition assay and for chromatographic analysis.

To determine in vitro conversion of the 2,4-diaminofolate antagonists to their 2,4-diamino-7-hydroxyfolate derivatives, necropsy liver specimens from man and rhesus monkey were purified for MTX oxidase by the method of Johns (13) through ammonium sulfate fractionation. In the case of monkey liver, rates of oxidation of the folate antagonists were determined by the marked hypsochromic shift of the 370-nm absorption maxima of MTX to 340 nm as previously described (14).

RESULTS

In studies with ^{3}H-MTX in man at conventional dose levels only trace amounts of non-MTX radioactivity have been observed (7); it was, therefore, of considerable interest that in the urine of primates treated with 3′,5′-7,9(n)^{3}H-MTX at 200 mg/kg, a significant amount of radioactivity was unassociated with dihydrofolate reductase inhibitory activity (fig 1). The chromatographic studies revealed a slowly migrating MTX-derived compound to be present in the monkey urine in increasing amounts after the 0–6-hour collection period. This compound was also present in the washed, protein-free supernatant fraction of the monkey kidney obtained at 24 hours (fig 2), as well as in the human urine examined from the collection period at 6–12 and 18–24 hours (table 1). This slowly migrating MTX-derived compound was some two orders of magnitude less effective an inhibitor of mammalian dihydrofolate reductase than was MTX. On the basis of chromatographic properties, ultraviolet and visible absorption spectra (fig 3), infrared spectra, weak inhibitory activity for dihydrofolate reductase (14), and limited aqueous solubility, the metabolite was identified as 7-hydroxy-MTX. The aqueous solubility of 7-hydroxy-MTX is some threefold to fivefold less than that of MTX over the pH range 5.0–7.0 (fig 4).

Histologic sections from the kidneys of monkeys with MTX-induced renal failure revealed dense material in the lumen of renal tubules after preparation with the Von Kossa stain. To determine if this material was, in fact, MTX or MTX derived, kidney slices from a monkey that had received ^{3}H-MTX 24 hours previously were autoradiographed (fig 5). These studies showed crystalline-like deposits within the tubules with exposed silver grains in the overlying emulsion. A protein-free supernatant from the same kidney tissue chromatographed on DEAE-cellulose revealed 77% of the ^{3}H-radioactivity to be associated with the metabolite (fig 2).

Partially purified monkey liver preparations catalyze the conversion of MTX to 7-hydroxy-MTX as evidenced by the characteristic change in ultraviolet and visible absorption spectra and the migration on DEAE-cellulose. The reaction rate can be measured by the standard spectrophotometric assay. The Michaelis constant for MTX oxidation by monkey liver MTX oxidase is on the order of 10^{-3} M, which is approximately tenfold greater than the Michaelis constant for the rabbit liver MTX oxidase (15). However, the rate of reaction of MTX oxidation is some three orders of magnitude slower with monkey enzyme than with rabbit enzyme. An unexpected finding was the ability of the monkey enzyme to catalyze the oxidation of aminopterin at the same rate as MTX; in most other species examined except the guinea pig, significant oxidation was not seen with aminopterin as substrate (9). With human liver preparations, 7-hydroxylation of MTX cannot be detected spectrophotometrically.

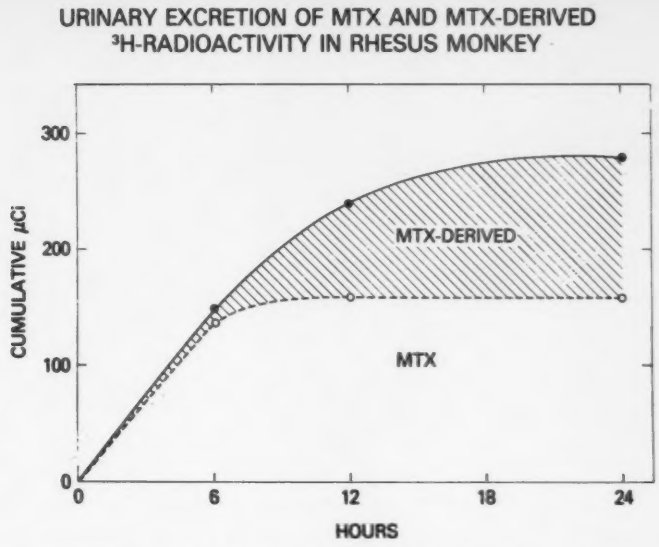

FIGURE 1.—Cumulative urinary excretion of MTX and MTX-derived metabolite in a rhesus monkey. A female rhesus monkey (6 kg) was given a 6-hr iv infusion of 3′,5′-7,9(*n*)³H-MTX (Dhom products, 10 microcuries/mmol) diluted with unlabeled MTX to a dose level of 200 mg/kg (546 microcuries) and a final specific activity of 148 microcuries/mmol. Urine was collected over time periods 0–6, 6–12, and 12–24 hrs. For each collection period the total radioactivity (in microcuries) was determined by liquid scintillation counting and the MTX concentration determined by inhibition of mammalian dihydrofolate reductase activity. The amount of radioactivity (in microcuries) associated with urinary MTX was calculated from the specific activity of 3′,5′-7,9(*n*)³H-MTX (148 microcuries/mmol). During the 24-hr period 49% of the total radioactivity administered was recovered in the urine.

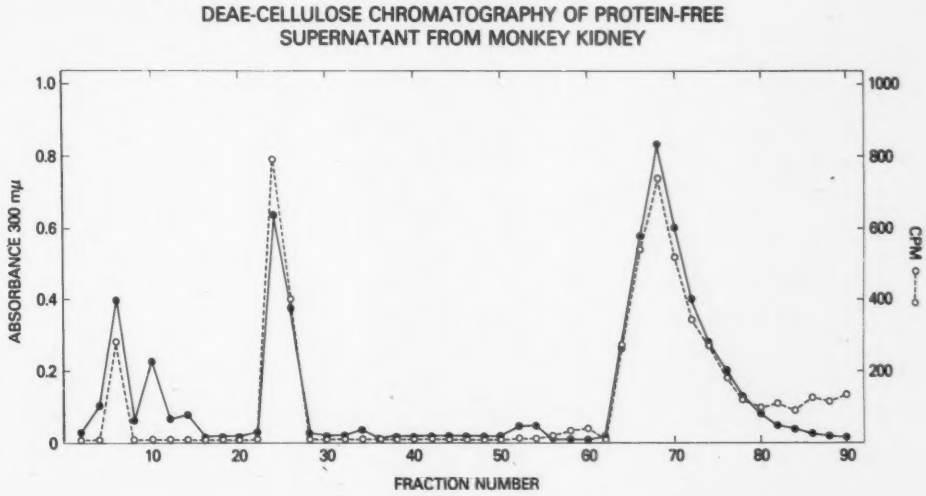

FIGURE 2.—DEAE-cellulose chromatographic elution patterns of MTX and 7-hydroxy-MTX in the protein-free supernatant fraction of a monkey kidney. A female rhesus monkey (6 kg) was given 3′,5′-7,9(*n*)³H-MTX at 200 mg/kg (546 microcuries) and killed 24 hrs after the infusion. A 1.98-g portion of kidney tissue was homogenized in 5.0 ml of 1% $NaHCO_3$. The homogenate was boiled for 5 mins and centrifuged at 25,000 × *g* for 30 mins. A 2-ml aliquot of the supernatant was diluted to a volume of 10 ml with distilled water and applied to a DEAE-cellulose column, and gradient elution was carried out as described in the text. ○ = absorbance at 300 nm; ● = ³H-radioactivity, cpm/0.1-ml eluate. The early peak of radioactivity (fractions 4–8) has been previously shown to be made up of cleavage products of MTX (8). The identities of the MTX and 7-hydroxy-MTX peaks (fractions 22–28 and 62–78 respectively) were established from the site of elution of standard reference compounds, from the ultraviolet and visible absorption spectra, and, in the case of MTX, from dihydrofolate reductase inhibition assay.

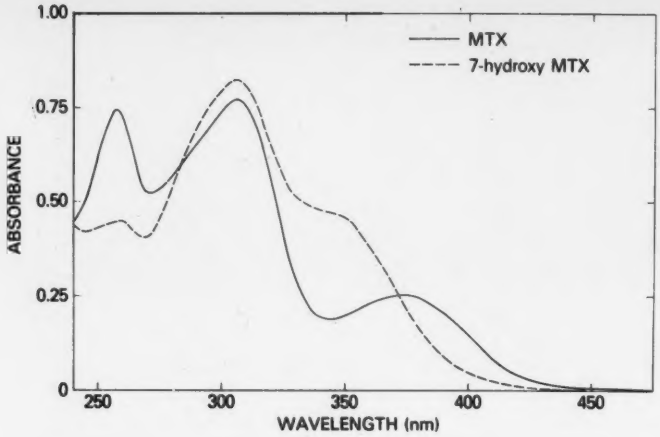

FIGURE 3.—Absorption spectra of MTX and 7-hydroxy-MTX at a concentration of 4.3×10^{-5} M in 0.02 M sodium phosphate buffer, pH 7.5.

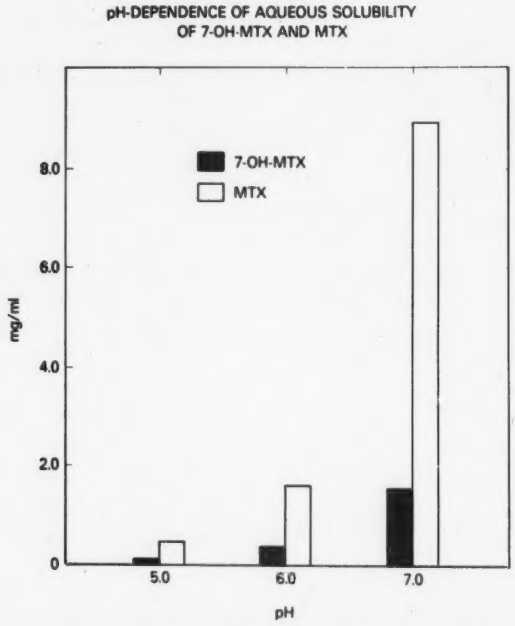

FIGURE 4.—pH-dependence of aqueous solubility of 7-hydroxy-MTX and MTX. Concentrations of dissolved 7-hydroxy-MTX and MTX were determined spectrophotometrically utilizing the molar extinction coefficient (7.0×10^3 M^{-1} cm^{-1}) at the isosbestic point of 370 nm after a 2-hr equilibration period in sodium phosphate buffer, 0.1 M, at 37°C.

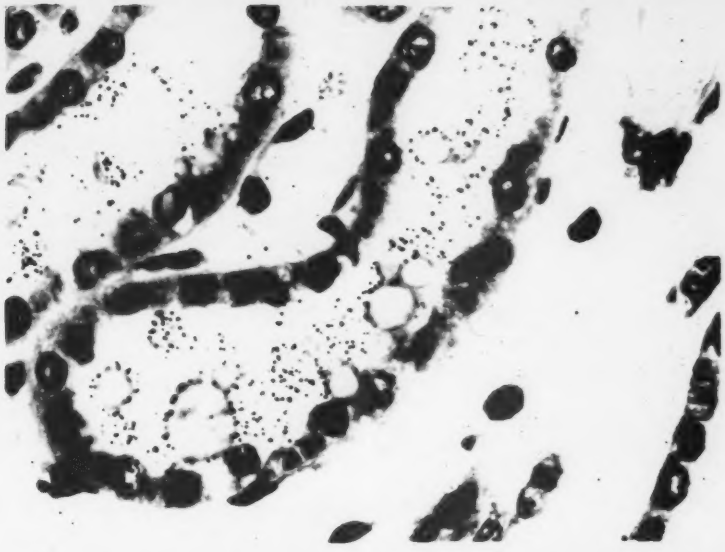

FIGURE 5.—Kidney autoradiograph, × 860, from a rhesus monkey killed 24 hrs after administration of 3',5'-7,9(*n*)^{3}H-MTX at a dose of 200 mg/kg (546 microcuries). The tissue, fixed in absolute alcohol, was cut in 5μ sections. The slides, dipped in Kodak Nuclear Track Emulsion NTB2 heated to 45°C, were exposed for 14 days, developed, and stained with hematoxylin and eosin.

DISCUSSION

The conversion of MTX to 7-hydroxy-MTX has not been described before in man and rhesus monkeys and does not appear to be a quantitatively important pathway when the drug is administered at what have been regarded as conventional dose levels. The recent clinical introduction of high-dose MTX, however, prompted us to initiate the present studies which were carried out at dose levels some fivefold to 2000-fold higher than earlier pharmacologic studies in man and 600-fold higher in monkeys (8,16). The unusual dose dependence for the generation of 7-hydroxy-MTX in vivo is likely due to both the low affinity of the parent drug for the enzyme catalyzing the oxidation and the slow rate of the reaction. At low or conventional dose levels of MTX, the 7-hydroxylation is not readily seen because of the quantitative predominance of other routes of disposition of the drug, ie, tight binding to dihydrofolate reductase and rapid urinary (16) and biliary[1] excretion. Only when these favored routes are saturated does the oxidative pathway compete effectively for the circulating drug.

Preparations of human liver yielded insufficient enzyme activity to be detected by the standard enzyme assay method. On the assumption that the reason activity cannot be measured is that the direct assay method lacks sufficient sensitivity, we are developing a radioisotopic assay based on the release of tritiated water as 3',5'-7,9(*n*)^{3}H-MTX is converted to 3',5'-9(*n*)^{3}H-7-hydroxy-MTX. In preliminary studies using this more sensitive assay method, low levels of MTX-oxidase activity have been detected in human liver.

Although the oxidation of MTX to 7-hydroxy-MTX decreases the potency of the drug as an inhibitor of mammalian dihydrofolate reductase by some 200-fold, the metabolite, because of its very limited solubility, may contribute to the syndrome of renal failure associated with high-dose MTX therapy. Only in those monkeys that developed renal failure were crystalline-like deposits detected within the renal tubules. This material was identified by the autoradiographic studies as MTX derived. Analysis of the monkey kidney tissue indicated that at least three fourths of the labeled drug within the kidney is in the form of the metabolite, 7-hydroxy-MTX. Since both MTX and 7-hydroxy-MTX are of limited aqueous solubility, especially in the acid pH range, both compounds probably contribute to the formation of the precipitated material.

[1] Azarnoff D, and Haas C. Personal communication.

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Antimetabolites in the Central Nervous System

Joseph H. Burchenal[1,*]

In introducing the subject of the clinical use of antimetabolites in leukemia and other neoplastic diseases involving the central nervous system it might be worthwhile to review briefly the early development in the field of intrathecal antimetabolite therapy.

Drawing on the techniques that were in general use for the assay of antibiotics, a simple disc method for the determination of levels of methotrexate[2] (MTX) in blood and urine was developed in our laboratories in 1951 using the pour plate seeded with *Streptococcus faecalis* (1). This had advantages over some of the other techniques, such as the tube method used by Swendseid et al (2), in that (*a*) sterility of blood and urine samples was not required, (*b*) colored or cloudy solutions or suspensions did not interfere with the accuracy of the test, and (*c*) very small blood and urine samples could be used if desired, 0.5 ml of serum being adequate for replicate determinations. With this technique, studies of serum and urine levels after oral administration in normal volunteers and in one patient with impaired renal function were done. As shown in figure 1, MTX, dissolved in water and taken on an empty stomach, was rapidly absorbed and equally rapidly excreted in volunteers with normal kidney function. On the other hand, in the patient with impaired renal function, the serum level remained high for a relatively long period of time and actually was as high after 24 hours in the patient as it was after 2 hours in the volunteers. It was also demonstrated that MTX, [illegible] on a full stomach, was absorbed more slowly, never reached as high a level in the serum, and was excreted more slowly. This technique was also used by Fountain et al in 1953 (3,4) to assay the MTX content in various organs in the mouse at various times after administration of the drug. In these studies, they discovered the interesting and at that time somewhat unexplained fact that MTX levels in the liver after the first 24 hours remained at levels of 200–400 μg/g of wet tissue for as long as 8 months after the administration of MTX.

The availability of such a technique for MTX assay, plus the fact that children with acute leukemia in complete bone marrow remission developed central nervous system leukemia (5), suggested to Whiteside et al (6,7) that MTX was not penetrating to the central nervous system in adequate concentrations (fig 2) and that it might profitably be injected directly into cerebrospinal fluid. Working in Frederick S. Philips' laboratory (Sloan-Kettering Institute), Whiteside injected MTX intracisternally in dogs and followed cerebrospinal fluid and blood levels by the disc assay method in our laboratory (fig 3). He demonstrated that dogs tolerated up to 0.5 mg/kg of MTX intrathecally and yet exhibited megaloblastosis and depression in the nucleated cell count in the bone marrow. MTX remained in high concentration for prolonged periods of time in the cerebrospinal fluid and gradually diffused into the blood in sufficient quantities to cause severe systemic toxicity. A single intrathecal dose of 1.8 mg/kg caused death from marrow depression and damage to gastrointestinal epithelium 6 days after administration. After establishing a tolerated dose in dogs, Whiteside then administered small doses intrathecally to patients with leukemic meningitis. Intrathecal instillation in these patients yielded extremely high cerebrospinal fluid levels and prolonged serum levels of MTX (fig 4). Five patients with neurologic symptoms due to leukemia and lymphosarcoma improved markedly when given 0.1–0.5 mg/kg intrathecally. The neurologic remissions began about 1 week after instillation and lasted about 6 weeks (7). These pharmacologic studies of Whiteside (6,7), reported in 1957 and 1958, ushered in the whole era of intrathecal MTX in the treatment and later in the prophylaxis of central nervous system leukemia.

Cytosine arabinoside was first used intrathecally by Bromylee et al in 1968 (8) and then independently by Band et al (9) and Wang and Pratt (10) in patients with meningeal leukemia and by Ziegler et al (11) in Burkitt's tumor. It is now being used prophylactically for acute leukemia. MTX and cytosine arabinoside are, at the present time, the two most important antimetabolites administered directly into the central nervous system.

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[1] Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

**Reprint requests to:* Joseph H. Burchenal, MD, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

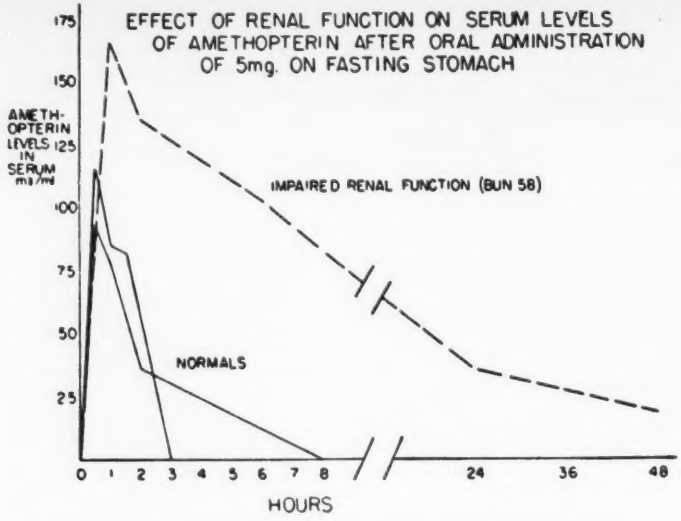

FIGURE 1.—Pharmacokinetics of MTX following oral administration in normal adults (solid line) and in patients with impaired renal function (broken line).

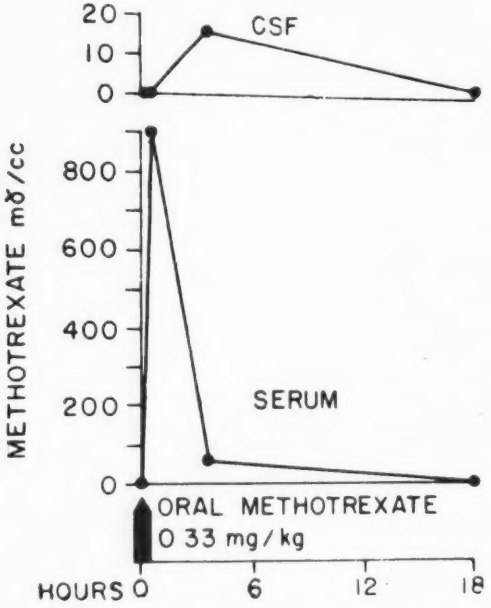

FIGURE 2.—Distribution of MTX in the cerebrospinal fluid compared to serum following oral administration in a child with acute leukemia.

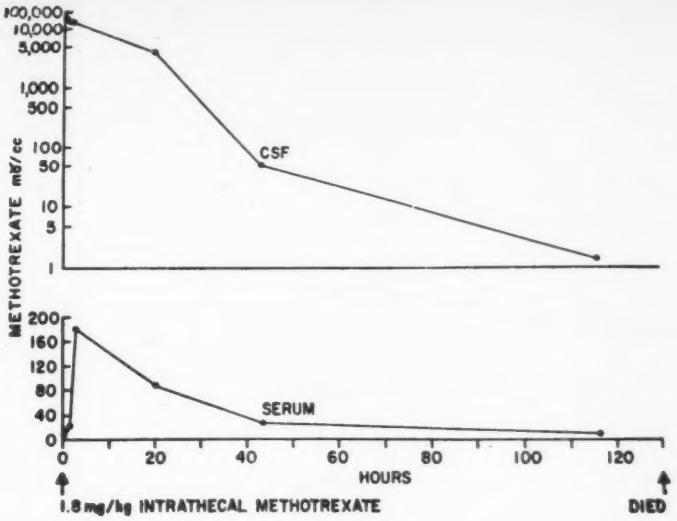

FIGURE 3.—Distribution of MTX in the cerebrospinal fluid compared to serum following intrathecal administration in the dog.

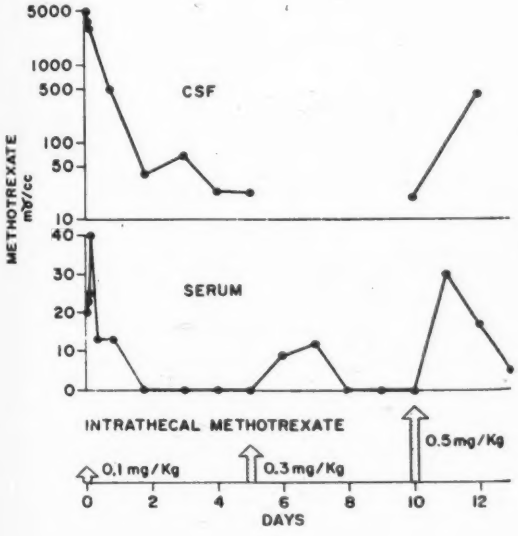

FIGURE 4.—Comparison of the distribution of MTX in cerebrospinal fluid and serum following several intrathecal administrations in a patient with leukemic meningitis.

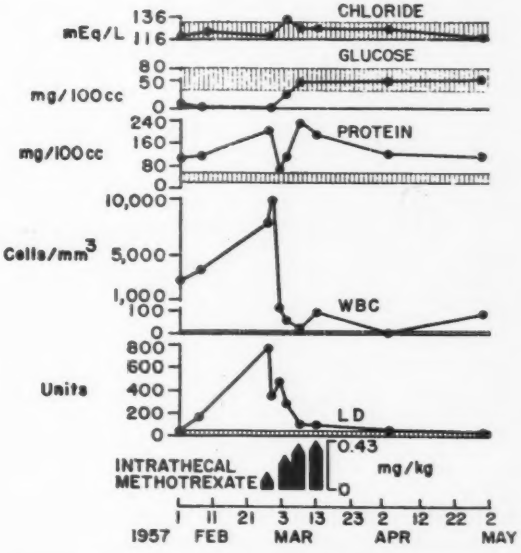

FIGURE 5.—Effects of intrathecal methotrexate administration in patient with leukemic meningitis.

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Antimetabolites in the Prophylaxis and Treatment of Central Nervous System Leukemia [1]

Mahroo Haghbin [2,*]

SUMMARY

The results of the administration of two antimetabolites, methotrexate (MTX) and cytosine arabinoside (Ara-C), for the prevention and treatment of established central nervous system (CNS) disease in children with acute lymphoblastic leukemia are discussed. Two protocols (L-2 and L-10) for the management of patients with acute lymphoblastic leukemia have been developed. In the L-2 protocol, prophylaxis consists of repeated intralumbar injections of MTX alone, and in the L-10 regimen, both MTX and Ara-C are administered; for the patients with an initial leukocyte count of ≥ 25,000/mm^3, the two drugs are given intraventricularly instead of by the usual intralumbar route. In the treatment of established CNS leukemia, intralumbar MTX and Ara-C in addition to CNS irradiation are employed; for maintenance, periodic intraventricular MTX injections are given. Of the 70 children receiving the L-2 protocol, four developed CNS leukemia and in a fifth patient, CNS and bone marrow relapse were concurrent. Among the 31 children receiving the L-10 regimen, CNS disease has been observed in only one child. Of the five children treated for the established CNS leukemia, recurrence was observed in two at 19 and 29 months after remission; the other three remain in remission for 2, 15, and 39 months, respectively.

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The first intrathecal administration of an antimetabolite, aminopterin, for the treatment of central nervous system (CNS) leukemia was reported in 1954 (1). Intrathecal methotrexate (MTX),[3] another folic acid antagonist, was shown to control the manifestations of CNS leukemia in 1958 (2), and since then, it has become a standard treatment for the neurologic complications of leukemia. Cytosine arabinoside (Ara-C),[4] given by the same route, has also been found to be a useful drug in the management of CNS leukemia (3,4).

With the increasing incidence of CNS disease, as the survival of patients with acute leukemia improved, attention was focused toward prevention of this complication. The earlier trials with the prophylactic administration of intrathecal MTX suggested that this technique can prevent or delay the development of CNS leukemia (5–7). Subsequent studies with and without the addition of CNS irradiation have established the efficacy of intrathecal MTX in the prophylaxis of CNS leukemia (8,9).

This paper discusses the results of prophylaxis and treatment of CNS disease with the two antimetabolites, MTX and Ara-C, in children with acute lymphoblastic leukemia (ALL) at Memorial Sloan-Kettering Cancer Center.

METHODS AND MATERIALS

Prophylaxis

Two protocols have been developed for the management of ALL in children (≤ 15 years of age), and

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[3] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-. Supplied by Lederle Laboratories, Pearl River, NY.

[4] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride. Supplied by The Upjohn Company, Kalamazoo, Mich.

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Induction:	Prednisone, vincristine, and daunorubicin, 4 wks
CNS Prophylaxis:	Intralumbar MTX, 3 doses
Consolidation:	Ara-C, 6-thioguanine, L-asparaginase, and BCNU, 9 wks
CNS Prophylaxis:	Intralumbar MTX, 1 dose
Maintenance:	6-Thioguanine, cyclophosphamide, hydroxyurea, daunorubicin, MTX, BCNU, Ara-C, and vincristine, 8 wks, to be repeated
CNS Prophylaxis:	Intralumbar MTX, 2 doses every 8 wks

FIGURE 1.—Outline of L-2 protocol for treatment of ALL.

in both of these protocols chemotherapy alone, without CNS irradiation, is employed for the prevention of CNS disease. The two protocols are identified as L-2 and L-10.

L-2 Protocol

The outline of the L-2 regimen is presented in figure 1; therapeutic details have been described previously (10). Prophylactic therapy consists of repeated intralumbar injections of MTX in a dose of 6.25 mg/m² (total dose not > 10 mg) beginning with the induction and continuing through the 3 years of the treatment course. A single dose of MTX is given on Day 4 of induction, followed by two doses at alternate-day intervals at the end of this phase and again by a single injection at the conclusion of consolidation. During the maintenance phase, patients receive two doses of intralumbar MTX on an alternate-day schedule, every 8 weeks. When the protocol was designed in 1969, preventive therapy was limited to the first two parts; with a revision in 1971, intralumbar injections were extended to the maintenance program.

Seventy-five previously untreated or minimally treated children were enrolled in the L-2 protocol. Seventy patients are evaluable for the study of the effectiveness of CNS therapy; 16 of these, who are the first entries in the protocol, did not receive intralumbar MTX injections in the maintenance phase until 1971.

Induction:	Prednisone, vincristine, and adriamycin, 4 wks
CNS Prophylaxis:	Intralumbar MTX, 3 doses
Consolidation:	MTX, Ara-C, 6-thioguanine, L-asparaginase, and cyclophosphamide, 16 wks
CNS Prophylaxis:	Intralumbar or intraventricular MTX, 2 doses; Ara-C, 3 doses
Maintenance:	Prednisone, vincristine, adriamycin, 6-mercaptopurine, MTX, actinomycin D, cyclophosphamide, and BCNU, 19 wks, to be repeated
CNS Prophylaxis:	Intralumbar or intraventricular MTX, 2 doses every 8 wks

FIGURE 2.—Outline of L-10 protocol for treatment of ALL.

Intralumbar MTX alternating with Ara-C
Insertion of Ommaya reservoir
Irradiation to neuroaxis, 600 rads
Intraventricular MTX, 2 doses every 8 wks
Systemic and CNS chemotherapy × 3 yrs

FIGURE 3.—Protocol for treatment of established CNS leukemia.

L-10 Protocol

This protocol, planned in 1973 (11), is outlined in figure 2. Preventive therapy for CNS leukemia begins with the induction and is continued through the 3-year treatment course. Intralumbar MTX (7 mg/m², total dose not > 10 mg) is given on Day 4 of induction and at the end of this phase using the same schedule as that of the L-2 protocol. Following the attainment of remission and at the beginning of the consolidation phase, a subcutaneous cerebrospinal fluid (CSF) Ommaya reservoir (12) is inserted by a neurosurgeon[5] for the patients with an initial peripheral wbc count of ≥ 25,000/mm³. All the subsequent CNS prophylactic treatment in this group is given intraventricularly via the reservoir. The intraventricular injection of MTX has been shown to provide a more reliable drug distribution in the CSF than the intralumbar route (13). An intraventricular injection of 6.25 mg/m² of MTX gives a ventricular CSF concentration of 2×10^{-4} M and with an intralumbar injection of the same dose, the ventricular concentration is variable and occasionally there is epidural and subdural leakage (13). It is expected that this technique will offer better protection in patients who are at a greater risk for CNS leukemia, namely those with a high wbc count at diagnosis. In addition to MTX, Ara-C (25 mg/m²) is also administered in the consolidation phase; two doses of MTX and three doses of Ara-C are given intralumbarly in the low wbc group and intraventricularly in the high wbc group. During maintenance, two intralumbar or intraventricular injections of MTX are given on alternate-day schedules every 8 weeks.

Thirty-one previously untreated or minimally treated children with ALL have been entered in the L-10 protocol. Nine of these patients had wbcs > 25,000/mm³ at diagnosis, and an Ommaya reservoir for each was inserted after remission induction.

Established CNS Leukemia

The protocol for the management of established CNS leukemia is a combination of antimetabolite

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therapy, MTX and Ara-C, plus CNS irradiation. The diagnosis of CNS leukemia is based on the presence of leukemic cells in the CSF and not pleocytosis. The outline of the protocol used for the first episode of CNS disease is shown in figure 3. Intralumbar MTX (7 mg/m²) and Ara-C (25 mg/m²), alternating every 2 days, are given until the CSF is clear. To maintain the CNS remission, intraventricular MTX, two doses every 2 months, is administered via an Ommaya reservoir, which is implanted after the elimination of the leukemia cells from the CSF. Irradiation to the neuroaxis (600 rads) is undertaken following insertion of the subcutaneous CSF reservoir. Systemic chemotherapy is resumed if CNS relapse is observed after the cessation of antileukemia treatment.

Technique of Intralumbar and Intraventricular Injections

Commercially available MTX and Ara-C were administered in these studies. Initially, undiluted 5-mg vials of MTX which contained methylparaben and propylparaben as preservatives were used. After the manufacturer substituted benzyl alcohol for these compounds, the 50-mg vials of MTX were diluted with distilled water, without preservative, to a concentration of 2.5 mg/ml before injection. For Ara-C, the 100-mg vials were mixed with the same diluent to a concentration of 20 mg/ml. The diluted preparations of MTX and Ara-C were filtered prior to the administration. Lumbar punctures were performed with a 20- or 22-gauge spinal needle in the lateral position. The puncture of the Ommaya reservoir was carried out in the supine position with a short 20- or 22-gauge spinal needle. After obtaining samples of CSF for cytologic and chemical analysis, the syringe containing MTX or Ara-C was connected to the spinal needle; 5–8 ml of CSF was drawn in the syringe for admixture with the medication and re-injected slowly. Administration of medication was avoided if the tap was traumatic and CSF blood tinged. Patients were asked to remain in supine or prone position for 1 hour following the lumbar tap. In older children, who tended to have more frequent and longer lasting reactions, the prone position seemed to decrease the incidence of post-lumbar puncture headache. No recumbent position was required after intraventricular injections. After the termination of the 3-year chemotherapy course, the CSF is examined for the presence of leukemic cells every 2 months for the first 2 years, and every 3 months subsequently.

RESULTS

Prophylaxis

L-2 protocol.—Of the 70 children who were treated with this regimen, four developed CNS leukemia while still in hematologic remission. Three of these children were among the first entries in the protocol and did not receive intralumbar MTX in the earlier part of their maintenance program. The relapses were detected on routine lumbar punctures in asymptomatic patients. The intervals from the initial diagnosis of leukemia to the development of CNS disease were 10, 32, 42, and 55 months. In one child, CNS leukemia and bone marrow relapse occurred concomitantly at 4½ months while the patient was symptomatic. The period of observation for the other children ranged from 33 to 73 months.

L-10 protocol.—Of the 31 children in this program, CNS disease has been observed in only one patient at 21 months following the diagnosis of leukemia. This child was asymptomatic when CNS leukemia was detected on a routine CSF examination. The period of observation for the other children ranged from 1 to 30 months.

The characteristics of the five children with CNS disease at the time of initial diagnosis of leukemia are recorded in table 1.

Established CNS Leukemia

Of the five children who were treated for CNS disease, two relapsed at 19 and 29 months, respectively, following remission and three remain disease-free for 2, 15, and 39 months. The child with the longest remission has not had chemotherapy, systemic or CNS, for 4 months.

Toxicity

A total of 2362 intralumbar injections for prophylactic (2276 with MTX and 63 with Ara-C) and therapeutic (17 with MTX and six with Ara-C) purposes have been given. No life-threatening reactions or neurologic deficits have been encountered. The

TABLE 1.—Characteristics of children with CNS leukemia at initial diagnosis

Age (yrs)	Sex	Wbcs/mm³	Platelets/mm³
1.5	F	25,800	10,000
1.5	M	9100	27,000
2.5	M	11,000	108,000
4.5	F	40,000	22,000
15	M	4100	116,000

TABLE 2.—Prophylactic regimens with intrathecal therapy for CNS leukemia

Study group or protocol	Drug and schedule	Rate of CNS leukemia relapse*	Ref No.
Medical Research Council	MTX, 8 mg/m² weekly × 5	18/122	15
Protocol 06 LA 66-Paris	MTX, 12 mg/m² at 0.5, 1, 2, 4, and 7 mos, then monthly	33%	16
Acute Leukemia Group B, protocol 7111	MTX, 12 mg/m² × 6	18%	17†
Children's Cancer Study Group	MTX, 12 mg/m² × 6	17/40	18
	wbcs > 20,000/mm³	6/96	
	wbcs < 20,000/mm³		
L-2	‡	4/70	
L-10	‡	1/31	

*No. of patients relapsing/total No. of patients.
†*See* footnote 6.
‡*See* figs 1 and 2.

observed reactions were limited to the manifestations of chemical meningitis and are as follows (in order of frequency): headache, backache, vomiting, fever, and leg pain. In no instance have the adverse effects necessitated the cessation of therapy. The duration of these symptoms varied from 1 to 5 days with the usual course being 1–2 days. There was no difference in the intensity and severity of reactions between MTX and Ara-C. Our previously reported incidence of post-intrathecal MTX headache (35%), backache (22%), vomiting (13%), fever (10%), and leg pain (7%) (9) has not changed despite an increase in the number of injections. A temporary rise in the frequency of these reactions was encountered when undiluted MTX with benzyl alcohol as a preservative was used. The problem was remedied by diluting the drug with distilled water without a preservative.

A total of 245 intraventricular injections for prophylactic (88 with MTX and 27 with Ara-C) and therapeutic (108 with MTX and 22 with Ara-C) purposes have been given. The type of side effects are similar to those for intralumbar injections, except for the absence of backache and leg pain. Repeated intraventricular administration of chemotherapy for as long as 3 years has not been associated with any adverse effects. The only complication was infection of the reservoir, which occurred in a child 20 months after its implantation for the treatment of CNS leukemia. In this case, the reservoir was removed following the detection of infection. A high incidence of pleocytosis has been reported with the prophylactic intrathecal administration of MTX (14). Of the 2339 intralumbar injections of antimetabolites for prophylaxis, 972 were followed by a second injection, and analysis of the CSF in these instances showed pleocytosis in 57 cases. Pleocytosis was defined as a cell count $\geq 10/mm^3$. Of the 115 intraventricular injections, 35 were followed by a second injection; analysis of the CSF fluid in these instances showed pleocytosis in four cases. In the combined 61 cases, the median cell count was $30/mm^3$ with a range of $10–559/mm^3$ and was $> 100/mm^3$ in only nine patients. The cells on cytocentrifuge preparation were mainly mononuclear, but in some samples, a significant increase of up to 60% of polymorphonuclear leukocytes was seen. In two instances, eosinophil counts of 20% and 47% were observed. The intensity of the side effects correlated with the pleocytosis, but not every symptomatic patient showed an increase in the CSF cell count.

DISCUSSION

The results of prophylactic therapy in these studies indicate that CNS leukemia can be controlled with repeated instillation of antimetabolites in the CSF. Before 1969, when no preventive treatment for CNS leukemia was employed in our center, 50% of the children with ALL developed CNS disease within the first year after diagnosis. The most widely used technique for prophylaxis is currently the combination of CNS irradiation and intrathecal MTX, first introduced by the group at St. Jude Children's Research Hospital (Memphis, Tenn) (8). Administration of intrathecal MTX alone, for the purpose of CNS prophylaxis, has been used in a number of studies (15–18); their results are listed in table 2. Systemic treatment differs widely in these regimens, and prophylactic therapy has not been given in the maintenance program except for the protocol 06 LA 66-Paris (16). In a study conducted by the Children's Cancer Study Group (18), no significant difference in the rate of CNS relapse was found between intrathecal MTX alone and CNS irradia-

tion for children with initial wbcs < 20,000/mm³ at diagnosis. However, the frequency of CNS relapse for those with wbcs > 20,000/mm³ was higher in the group who received intrathecal MTX alone. The results of the Acute Leukemia Group B (protocol 7111) (17) show an 18% relapse rate with intrathecal MTX alone,[6] while our results are comparable to those of the St. Jude group employing irradiation (study VII, five CNS relapses among 94 patients) (19).

In the treatment of established CNS leukemia, aminopterin had a limited trial in both maintained and unmaintained regimens (20,21). The mean duration of unmaintained remission with a dose of 0.1 mg/kg was 5.9 months (21). Intrathecal MTX, 0.25–0.5 mg/kg, was reported to produce a mean remission duration of 5 months (22). Intrathecal Ara-C, 5–70 mg/m², has yielded short remissions with a median of only 1 month (3). A dose of 12 mg/m² of intrathecal MTX given every 5–7 days until the normalization of CSF, and subsequently every 8 weeks as maintenance, has produced a median remission duration of 240 days compared to 106 days with an unmaintained schedule and 216 days with the neuroaxis irradiation (23). The combination of intrathecal MTX in a dose of 15 mg/m² with the same dose of hydrocortisone, administered every 4–5 days until the normalization of CSF and thereafter every 8 weeks, has lengthened the duration of CNS remission to a median of 317 days; the addition of Ara-C, 30 mg/m², to this combination has improved the median remission to 460 days (24). These studies point to the effectiveness of maintenance intrathecal therapy in the control of overt CNS leukemia. Although our results with the intraventricular maintenance regimen are limited to only five patients, the duration of remissions is impressive.

Intrathecal administration of MTX has been associated with a spectrum of serious toxicities such as paralysis (25), encephalopathy and dementia (26), and death (27). In these cases, treatment was therapeutic and not prophylactic. Intrathecal therapy with Ara-C has also been associated with severe neurologic deficit (25). Serious life-threatening toxicities of intrathecal MTX have been found to be related to the high concentration of the drug in the CSF (28). The elevated CSF level of the drug is attributed to delayed elimination or increased dosage (28). The contributory factors are assumed to be the presence of meningeal leukemia, which interferes with the transport of MTX from the CSF to the systemic circulation, and age of the patient. In older patients, intrathecal-dosage calculation based on body surface area can lead to excessively high doses (28). The absence of serious adverse effects in our series, in both intralumbar and intraventricular therapy, is perhaps explained by the lower dose of MTX (6.25–7 mg/m²) with the total dose not > 10 mg, as compared to the commonly used dose of 12–15 mg/m². There are no controlled clinical studies to compare the efficacy of low- versus high-dose MTX in the control of CNS leukemia. It has been speculated that sustained elevation of MTX in the CSF may lead to encephalopathy because of folate deficiency (26).

Intrathecal administration of MTX, unlike Ara-C, which upon diffusion in the plasma is rapidly deaminated to therapeutically inactive arabinosyl uracil with no hematopoietic effect (3), can cause bone marrow and gastrointestinal toxicity (29,30). When MTX is given intrathecally, it acts as a "depot," with plasma concentrations lasting twice as long as after an identical oral or iv dose (30). Citrovorum factor rescue has been employed for protection from systemic toxicity of intrathecal antifolate compounds (20,24,29).

At present, there is no systemic drug that can replace the intrathecal therapy for the prevention or control of CNS leukemia. Hopefully, with further studies on the pharmacokinetics of intralumbar or intraventricular administration of antimetabolites or other agents, optimum doses and schedules will be developed.

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Natural Resistance to Methotrexate in Human Acute Nonlymphocytic Leukemia [1,2]

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SUMMARY

Eight patients with various forms of acute nonlymphocytic leukemia with blast cell counts of >10,000/mm³ were treated by a single injection of methotrexate (120 mg/m²) followed by citrovorum factor rescue 36 hours later. All patients showed a decrease in the blast cell count that varied from 0.12 to > 4 logs, reaching a nadir at 3–5 days. Patients with < 1 log of cell-kill (five patients) were characterized by recovery of DNA synthesis in peripheral blast cells measured by ^{3}H-deoxyuridine incorporation into DNA at 24 hours after methotrexate. In addition, dihydrofolate reductase enzyme activity (total enzyme) was elevated threefold to fourfold over pretreatment values at 48 hours. In three patients with > 2 logs of cell-kill, no increase in total dihydrofolate reductase was measurable at 48 hours; two of these three patients had a slower clearance of plasma methotrexate than the other patients. Intracellular methotrexate levels were approximately the same in these two groups of patients, and decreased as plasma levels decreased. These observations indicate that after treatment with a high pulse dose of methotrexate, acute nonlymphocytic leukemia cells had a rapid influx of methotrexate and this level was sufficient to completely inhibit dihydrofolate reductase and DNA synthesis for several hours. As plasma levels decreased, intracellular methotrexate also rapidly decreased, presumably because of a rapid efflux. Those patients who had persistently high plasma levels because of borderline renal impairment and whose leukemia cells synthesized dihydrofolate reductase slowly had the greatest fall in peripheral blast count. Those patients whose cells had a more rapid synthetic rate for dihydrofolate reductase (as estimated by the increase in dihydrofolate reductase at 48 hours) were able to synthesize sufficient free dihydrofolate reductase, as plasma levels and intracellular levels of methotrexate decreased, to overcome the methotrexate block and then resume DNA synthesis at 24–36 hours. These studies suggest that the optimal way to utilize methotrexate in acute nonlymphocytic leukemia is by high-dose infusion sufficient to maintain blood levels of methotrexate $> 2 \times 10^{-6}$ M for the maximum possible time, ie, 36–42 hours, followed by "rescue" of normal cells with citrovorum factor, L-asparaginase, or carboxypeptidase G_1.

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A detailed understanding of methotrexate action in human leukemia cells is still not available despite considerable knowledge that has been accumulated on this subject (1–3). Although the major target of this inhibitor appears to be dihydrofolate reductase, other target sites have been suggested, in particular thymidylate synthetase (4,5).

In this report, we have attempted to correlate the pharmacologic and biochemical events that occur after methotrexate administration in patients with

[1] Supported by Public Health Service (PHS) grants CA-08010 and CA-08341 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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acute nonlymphocytic leukemia with the resultant blast cell decrease in order to understand "natural" resistance or sensitivity to methotrexate.

MATERIALS AND METHODS

Patients.—Patients with acute nonlymphocytic leukemia with peripheral blast cell counts of $> 10{,}000/mm^3$ were studied in the chemotherapy research center after informed consent was obtained for the studies and treatment program. The age and characteristics of the patients with regard to leukemia type are presented in table 1.

Measurement of ^{3}H-deoxyuridine incorporation into DNA.—Leukocytes were isolated from heparinized blood by dextran sedimentation (6), recovered from the supernatant by centrifugation, washed, and resuspended in Eagle's basal medium with Earle's salts (GIBCO, Grand Island, NY) containing 10% horse serum and 200 units of sodium heparin/ml. The cells were suspended at a concentration of $5–15 \times 10^6$ cells/ml. One-ml aliquots were distributed into flasks containing either saline or methotrexate at various concentrations and incubated in a shaking water bath at 37°C for 30 minutes. ^{3}H-deoxyuridine was then added to a concentration of 9×10^{-7} M, and at 5-minute intervals, 0.2-ml portions were removed from each flask and the DNA precipitated by the addition of 5 ml of ice-cold 10% perchloric acid. After allowing these tubes to stand overnight at 4°C, the tubes were centrifuged and the pellets were washed three times with 10-ml portions of ice-cold 10% trichloroacetic acid. After the pellets were dissolved in 0.4 ml of NCS[5] Tissue Solubilizer (Amersham/Searle, Arlington Heights, Ill), 15 ml of scintillation fluid was added and the radioactivity was determined using a Beckman scintillation counter.

Measurement of dihydrofolate reductase activity.—Dihydrofolate reductase was assayed as previously described (7). Protein was determined by the Biuret procedure and the specific activity is expressed as μmols of product formed/mg of protein/hour at 37°C.

Measurement of methotrexate in plasma and leukocytes.—Plasma methotrexate concentrations were measured after appropriate dilutions with normal saline by the dihydrofolate reductase inhibition method, using a purified L1210 dihydrofolate reductase as the enzyme source (7,8). Intracellular drug concentrations were measured after boiling isolated leukocytes suspended in saline for 3 minutes and measuring inhibitor activity in the supernatant by the enzyme method (8).

[5]Trademark of Nuclear-Chicago Corporation.

RESULTS

Eight patients diagnosed as having acute nonlymphocytic leukemia with blast cell counts of $\geq 10{,}000/mm^3$ were treated with a single dose of methotrexate (120 mg/m^2) followed by citrovorum factor[6] rescue 36 hours later (table 1). The decrease in the peripheral blast cell count, expressed as log cell-kill, varied from 0.12 to > 4 logs. The nadir of the blast cell decrease occurred on Days 3 or 4 for all of the patients studied; patients were then (Day 5) treated with combination chemotherapy utilizing adriamycin and cyclophosphamide[7] followed by methotrexate and cytosine arabinoside[8] (except for patient 8). The results of this treatment program will be reported in detail later. It should be noted, however, that five of the seven patients treated with the entire protocol achieved a complete remission. The role of the methotrexate injection in producing the remission cannot yet be assessed because of the small number of patients. Patients who had a > 1 log cell-kill are referred to as "sensitive" to methotrexate (patients 6–8), while patients whose peripheral blast cells decreased to < 1 log after methotrexate are referred to as being "resistant" (patients 1–5).

Effect of methotrexate on ^{3}H-deoxyuridine incorporation in vitro (pretreatment).—The rate of incorporation of ^{3}H-deoxyuridine into DNA in the absence and in the presence of three concentrations of methotrexate was measured in blast cells obtained from these patients prior to treatment (fig 1). No correlation between "response" and the degree of inhibition of DNA synthesis was noted; all patients (except for patient 2) showed approximately equivalent sensitivity to methotrexate at the dose levels tested. These data confirm previous data obtained from studies with acute lymphoblastic leukemia and acute myelocytic leukemia patients treated with methotrexate and citrovorum factor, that also failed to show any correlation between this in vitro sensitivity test and subsequent response in vivo (9). These data also indicate that uptake of methotrexate does not differ markedly in these patients, since sufficient intracellular drug concentration is achieved under these short-term in vitro conditions to markedly inhibit DNA synthesis at 2×10^{-6} M or

[6]Citrovorum factor: NSC-3590; CAS reg. No. 6055–86–5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

[7]Adriamycin: NSC-123127; CAS reg. No. 23214–92–8.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[8]Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

TABLE 1.—Acute myelocytic leukemia patient treatment profile

Patient No.	Diagnosis	Patient age/sex	Blast cells: Before treatment (cells/mm³)	Blast cells: After methotrexate (cells/mm³)	Blast cells: Day of nadir after methotrexate	Log-kill	Remission*
1	Hypoplasia × 7 mos, acute myelocytic leukemia	62/F	17,000	11,000	3	0.19	0
2	Acute myelocytic leukemia	20/M	15,200	11,500	3	0.12	CR
3	Acute myelocytic leukemia (2 alkylating agents, X-ray therapy?)	52/F	40,000	9000	3	0.65	0
4	Polycythemia vera, acute myelocytic leukemia	64/M	37,000	8200	3	0.65	CR
5	Acute myelocytic leukemia	42/F	13,000	2500	4	0.72	CR
6	Chronic myelocytic leukemia, blast crisis	46/F	10,000	100	5	2.0	CR
7	Acute myelocytic leukemia	59/M	200,000	425	5	2.67	CR
8	Chronic myelocytic leukemia, blast crisis	63/M	55,000	0	4	>4	Not on protocol

*CR = complete remission.

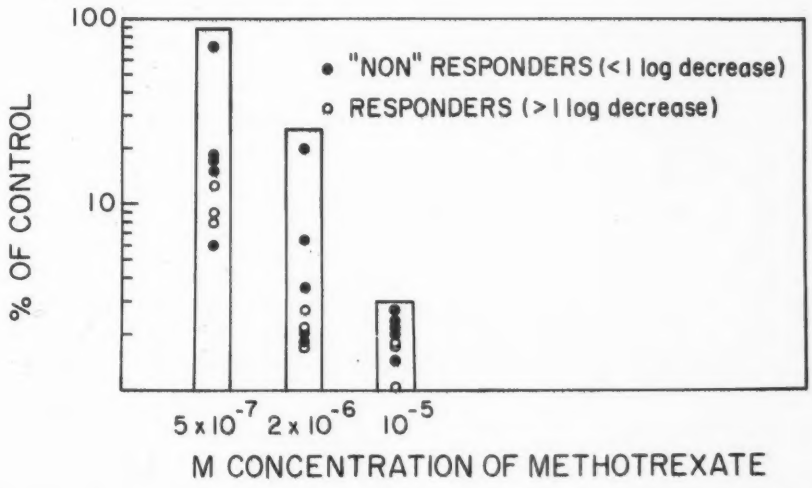

FIGURE 1.—Effect of increasing concentrations of methotrexate on incorporation of ^{3}H-deoxyuridine measured in patient cells incubated in vitro prior to initiation of therapy (see Materials and Methods). Control values refer to ^{3}H-deoxyuridine incorporation in the absence of methotrexate.

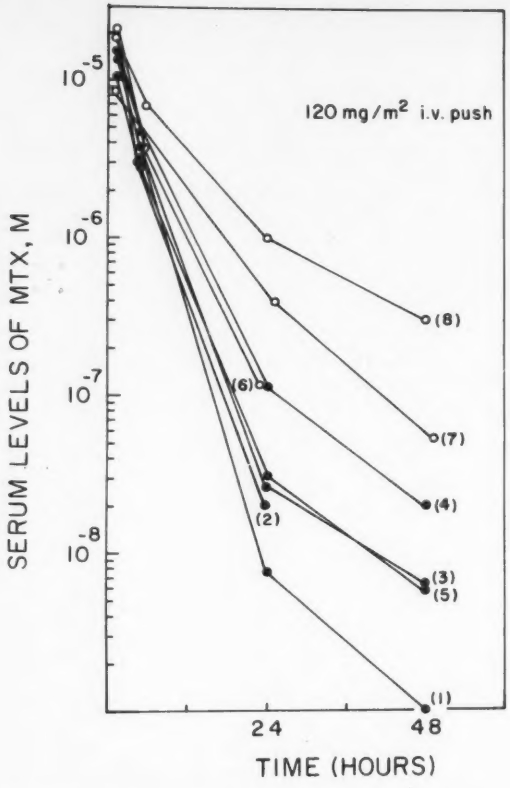

FIGURE 2.—Serum levels of methotrexate in the 8 patients studied following a single iv push of methotrexate (120 mg/m²). Levels were determined after appropriate dilution of serum with saline utilizing the enzyme inhibition assay (8). The numbers refer to the patients studied (table 1).

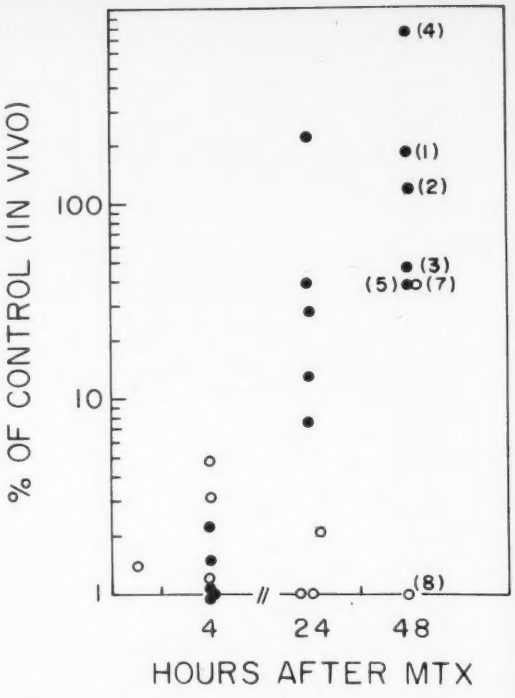

FIGURE 3.—Inhibition of ^{3}H-deoxyuridine in vivo in cells tested in vitro at intervals following methotrexate administration. After separating leukocytes from erythrocytes by sedimentation, the cells were resuspended in Eagle's medium + 10% horse serum for 15 mins at 37°C; then ^{3}H-deoxyuridine was added. Incorporation of ^{3}H-deoxyuridine into DNA was measured over the next 40 mins (*see* Materials and Methods). The numbers refer to the patients studied (table 1).

greater (except for patient 2, upper point in graph for each concentration).

Relationship of dihydrofolate reductase activity to methotrexate responsiveness.—As has been noted previously (10,11), the pretreatment level of the methotrexate-sensitive enzyme, dihydrofolate reductase, also did not correlate with responsiveness (table 2).

Serum clearance of methotrexate.—The serum levels achieved after the iv injection of 120 mg/m² of methotrexate in the eight patients are shown in figure 2. A wide variation in serum levels between patients was noted. The two patients with the highest levels (patients 7 and 8) also had elevated serum creatinine levels (2.6 and 2.1 respectively), and despite citrovorum factor therapy as described, were the only patients who had a toxic reaction from this methotrexate regimen; the toxic effect consisted of mild mucositis, lasting for several days. These data re-emphasize the variability of methotrexate clearance between patients and again demonstrate the importance of renal function and monitoring of methotrexate serum levels when high-dose methotrexate is used.

Inhibition of DNA synthesis in vivo following methotrexate administration.—Following the injection of methotrexate, the resulting inhibition of DNA synthesis produced in vivo was estimated by removing blood from patients at 4, 24, and 48 hours (12 hours after citrovorum factor), and expressing the rate of inhibition of ^{3}H-deoxyuridine in these blast cells as a percentage of the control (pretreatment) ^{3}H-deoxyuridine rate. Figure 3 indicates that at 4 hours, all patients had markedly reduced rates of incorporation of ^{3}H-deoxyuridine into DNA (< 5%

TABLE 2.—Pretreatment levels of dihydrofolate reductase and presence of "free" intracellular methotrexate after methotrexate administration*

Patient No.	Time after methotrexate (hrs)				
	0	1	4	24	48
1	0.007†	+‡	+	+	±
2	0.011	+	+	±	−
3	0.016	+	+	+	+
4	0.021	+	+	+	+
5	0.008	+	+	+	+
6	0.018	+	+	+	+
7	0.022	+	+	+	+
8	0.027	+	+	+	+

*Dihydrofolate reductase activity in leukemic extracts was measured after lysis of cells suspended in 5 ml of saline by freeze thawing × 3. The complete assay mix contained in a final volume of 1 ml was: Tris buffer, pH 7.0, 100 μmols; KCl, 150 μmols; NADPH, 0.08 μmol; and leukocyte extract, 0.2 ml. Dihydrofolate, 0.02 μmol, containing 10 μmols of 2-mercaptoethanol, was added last to initiate the reaction. The presence of "free" methotrexate was determined by the addition of a known amount of L1210 dihydrofolate reductase to the complete system containing the wbc extracts; if inhibition of this activity occurred then "free" methotrexate was present.

†μmols/hr/mg protein, pH 7.0, 37°C.

‡Refers to presence of "free" methotrexate.

of control). At 24 hours, two distinct patterns were observed; one group continued to show a marked inhibition of DNA synthesis (patients 6–8), while the other group showed recovery to 8% of control or greater. At 48 hours (12 hours after citrovorum factor), all patients except for patient 8, showed recovery to 40% of normal or greater.

Intracellular levels of methotrexate and the presence of "free" or excess methotrexate.—The levels of intracellular dihydrofolate reductase were measured and the presence of excess methotrexate was estimated (table 2). Inhibition of dihydrofolate reductase and the presence of "free" methotrexate did not correlate well with inhibition of ^{3}H-deoxyuridine incorporation into DNA synthesis (fig 3); thus, DNA synthesis was resumed even in the presence of "free" methotrexate, assayed as described in figure 3.

Levels of intracellular drug following treatment with methotrexate.—Intracellular methotrexate levels achieved were also measured at the times indicated following methotrexate administration (table 3). All patients had high intracellular concentrations when plasma levels were high at 1 hour, with subsequent decrease in levels noted at 24 and 48 hours. However, there was no difference in the levels achieved between responders and nonresponders as defined, at any of the time periods measured.

Increase in dihydrofolate reductase activity following methotrexate administration.—Previous studies have demonstrated that dihydrofolate reductase levels increased in leukemic leukocytes, normal leukocytes, and erythrocytes following methotrexate administration, due mainly to an increase in enzyme-bound methotrexate (E-methotrexate) (12,13). A rough estimate of total enzyme activity (E plus E-methotrexate) may be estimated by dilution of cell extracts and measurement of dihydrofolate reductase at pH 8.5, where the E-methotrexate complex is dissociable (13,14). Increases up to sevenfold were noted at 48 hours after methotrexate; with the exception of patient 4, the increases noted were observed in all patients "resistant" to methotrexate (table 4). In contrast, there was no detectable enzyme activity when measured at pH 7.0, even at 48 hours, except for patient 2 whose level had returned to normal by 48 hours.

TABLE 3.—Intracellular concentration of methotrexate*

Patient No.	Time after methotrexate (hrs)		
	1	24	48
	(μmols/mg × 10^{6})		
1	3.2	2.1	1.2
3	4.1	4.0	1.8
4	7.0	2.3	1.1
5	4.7	3.4	2.6
7	11.0	6.1	1.0
8	5.8	4.3	2.6

*Methotrexate levels in leukocyte extracts were assayed as described in Materials and Methods.

DISCUSSION

The present studies were undertaken to further understand natural resistance to methotrexate of

TABLE 4.—Increase in dihydrofolate reductase 48 hrs after methotrexate*

Patient No.	Fold increase
1	2.7
2	7.0
3	3.7
4	0.6
5	2.5
6	0.6
7	0.2
8	0.8

*Dihydrofolate reductase activity was measured at pH 8.5 using the following assay mix: Tris HCl buffer, pH 8.5, 100 μmols; KCl, 150 μmols; NADPH, 0.08 μmol; leukocyte extract, 0.2 ml; and 0.05 μmol dihydrofolate containing 10 μmols of 2-mercaptoethanol (added last to initiate the reaction). Fold increase refers to the ratio of enzyme activity measured at this pH to pretreatment levels.

patients with acute nonlymphocytic leukemia. In this small series of patients (all with acute nonlymphocytic leukemia but of various etiologies and cell types), three of the eight patients studied demonstrated "sensitivity" as defined. Whether or not these patients would have achieved a complete remission with repeated courses of methotrexate and citrovorum factor was not tested in view of the success of current therapeutic regimens utilizing anthracycline drugs and cytosine arabinoside (15,16). By analogy with a previous study, however, an every 3–4-day schedule with this regimen might have been capable of achieving remission in this "sensitive" small subgroup (9). The lack of value of the short-term in vitro test utilizing ^{3}H-deoxyuridine sensitivity to methotrexate was again noted (9); whether this test can be made more predictive by prolonging the duration of incubation to reflect in vivo events is worthy of further investigation.

In a sense, it is somewhat unfortunate that two of the patients with the greatest decrease in blast cells after methotrexate also had higher blood levels of the drug, thus obscuring somewhat the role of the other factors considered. These results do emphasize the variability in blood levels produced by a uniform dose of methotrexate and the need for careful monitoring of methotrexate levels.

The use of ^{3}H-deoxyuridine to follow methotrexate administration is a useful biochemical correlate of the degree of methotrexate block (9). Recovery of DNA synthesis occurred in the naturally resistant group, even before citrovorum factor rescue was started. As the serum level decreased, intracellular methotrexate concentrations also decreased, indicating a rapid efflux process in these cells. In addition, in all of the resistant patients (except for patient 4), dihydrofolate reductase levels increased. The continued synthesis of dihydrofolate reductase by these cells, in the face of a decreasing intracellular pool of methotrexate, apparently resulted in sufficient "free" dihydrofolate reductase activity to allow resumption of DNA synthesis, as reflected by an increase in ^{3}H-deoxyuridine incorporation into DNA at 24 hours, and to allow the formation of tetrahydrofolate sufficient for continuation of thymidylate biosynthesis.

It would seem clear, therefore, that if patients with an acute nonlymphocytic leukemia are to be treated with methotrexate, a very high dose should be administered so as to maintain blood levels of $>10^{-6}$ M for 36 hours, and that ^{3}H-deoxyuridine incorporation and/or "free" methotrexate should be measured as a function of time to ensure > 90% inhibition of DNA synthesis during this time. Assuming that some normal marrow and gastrointestinal stem cells are not sensitive to this degree and duration of therapy (as has been noted in patients with head and neck cancer who can tolerate 1 g/m^2 of methotrexate over 36–42 hours followed by citrovorum factor rescue [17,18]), this program may produce the greatest cell-kill with the least effect on normal stem cells. However, the effects of subsequent courses of methotrexate and citrovorum factor would be less effective, since presumably those cells not killed by the first course of therapy would have (a) increased dihydrofolate reductase levels and (b) increased amounts of citrovorum factor on hand, similar to those events occurring in normal granulocyte precursors. In this circumstance, alternative "rescue" procedures utilizing L-asparaginase (19), carboxypeptidase G_1 (20), or thymidine (21) might be employed.

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High-Dose Methotrexate With Citrovorum Factor in Osteogenic Sarcoma—Progress Report II[1,2]

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SUMMARY

Vincristine (VCR), high-dose methotrexate (MTX), and citrovorum factor (CF) were administered to 12 patients with classic osteogenic sarcoma with local control. Seven patients (58%) are free of pulmonary metastases for 2+–3½+ years. With a second adjuvant program incorporating adriamycin (VCR, MTX, CF, and adriamycin), 16 of 20 patients are free of pulmonary metastases for 6+–18+ months. Five patients who developed pulmonary metastases were rendered free of disease by surgical resection. The VCR, MTX, and CF program was also administered at weekly intervals to eight patients with pulmonary metastases or unresected primary lesions. Two complete and one partial response were obtained in four patients with pulmonary metastases and three complete and one partial response were obtained in patients with primary lesions. This program was also administered in combination with radiation therapy to four patients who relapsed on conventional VCR, MTX, CF, and adriamycin therapy following surgical resection of pulmonary metastases. They remain free of recurrent disease for 2+–14+ months. There was no alteration in the incidence of toxicity when compared to earlier investigations. The results indicate that the VCR, MTX, and CF program has had a major impact on the management of osteogenic sarcoma.

[Cancer Treat Rep 61:675–679, 1977]

High-dose methotrexate (MTX) with citrovorum factor (CF) has had a major impact in the management of osteogenic sarcoma. The regimen is employed as adjuvant therapy in the treatment of metastatic disease and preparatory to surgery of the primary tumor (1–6).[4] This paper will describe recent innovations in the program and update results to December 1975.

Basic High-Dose MTX Regimen (Vincristine [VCR],[5] MTX, and CF) (Fig 1)

There has been no change in the earlier schedules and doses of VCR and MTX (4,5). VCR (2 mg/m², maximum 2 mg) was administered ½ hour prior to initiation of the 6-hour MTX infusion. The MTX was increased from 3000 to 7500 mg/m² at 2–3-week intervals. The dose of CF was standardized: 15 mg every 3 hours was administered iv for eight doses

[1]Supported in part by Public Health Service grant CA-06516 from the National Cancer Institute, and by grant RR-05526 from the Division of Research Facilities and Resources, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}-.

Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, N-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl}-, calcium salt.

[3]Children's Hospital Medical Center, Sidney Farber Cancer Center, Harvard Medical School, and Joint Center for Radiation Therapy, Boston, Mass.

**Reprint requests to:* Dr. Norman Jaffe, Sidney Farber Cancer Center, 44 Binney St, Boston, Mass 02115.

[4]Jaffe N, Frei E III, Traggis D, et al. Weekly high-dose methotrexate-citrovorum factor in osteogenic sarcoma: presurgical treatment of primary tumour and of overt pulmonary metastases. Manuscript submitted for publication.

[5]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

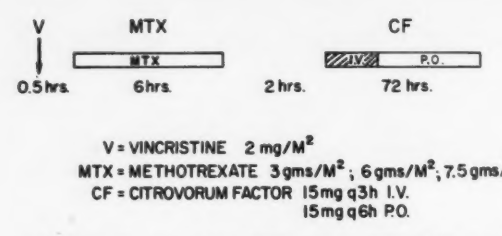

FIGURE 1.—Basic high-dose MTX regimen (VCR, MTX, and CF). VCR (2 mg/m²) is administered ½ hr prior to initiation of the MTX infusion. The first dose of MTX administered is 3000 mg/m², the second dose 2 wks later is 6000 mg/m², and the third dose 3 wks later is 7500 mg/m². The latter dose is then administered at 3-wk intervals for 18 mos.

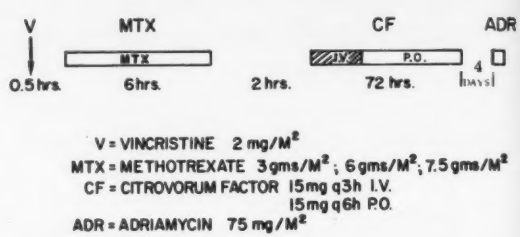

FIGURE 2.—VCR (2 mg/m²) is administered ½ hr prior to initiation of the MTX infusion. The first dose of MTX administered is 3000 mg/m², the second dose is 6000 mg/m², and the third dose is 7500 mg/m². Adriamycin (75 mg/m²) is administered 7 days after the second and subsequent MTX infusion. The interval between MTX courses is lengthened to 4 wks until the maximum cumulative dose of adriamycin (450 mg/m²) has been administered. VCR, MTX, and CF are then administered at 3-wk intervals. Total duration of treatment is 18 mos.

followed by 15 mg every 3 hours orally for eight doses. The first oral dose was started 3 hours after the last iv dose. Treatment was administered at 3-week intervals for 18–24 months. Entry in this study was terminated in June 1974.

Results (Table 1)

The regimen was administered to 20 patients as adjuvant therapy (4). Twelve patients had classic osteogenic sarcoma with local control and seven (58%) are free of pulmonary metastases for 2+–3½+ years. In the patients who relapsed, metastases were detected at 5, 7, 11, 12, and 19 months. (Surgical removal of isolated metastases has rendered one of these patients free of disease for 12+ months.) The latest metastasis was detected 1 month after the discontinuation of treatment with the appearance of a second (? primary) osteogenic sarcoma in the contralateral nonamputated femur. The patient was classified as a relapse although it is possible that the metastases were derived from the "new" tumor.

Among eight patients with non-classic osteogenic sarcoma (4), five are free of pulmonary metastases for 2+–3½+ years. Four had the parosteal variety which was treated by wide local excision rather than amputation.

MTX and Adriamycin[6] Regimen (VCR, MTX, CF, and Adriamycin)

This adjuvant regimen involves the administration of adriamycin at a dose of 75 mg/m² 1 week after each high-dose MTX treatment with the interval between MTX treatments extended from 3 to 4 weeks (fig 2). When the maximum cumulative dose of adriamycin has been attained (450 mg/m²), the triweekly schedule is reinstated.

Results (Table 1)

Twenty patients in whom local control was achieved have been treated for 6–18 months. Four have developed pulmonary metastases at 6, 6, 13, and 14 months respectively. These have been removed surgically. One additional patient developed a sclerosing bone metastasis in a rib and contralateral femur while the lungs remained free of disease. These lesions, which were removed, may represent a variety of multifocal sclerosing osteogenic sarcomas. One patient developed inguinal and iliac node metastases 6 months after initiation of treatment; the metastases probably occurred from drainage of the original tumor. It was, however, decided to retain this patient in the category of "local control." The nodes were removed surgically and weekly high-dose MTX and radiation therapy were administered (vide infra).

Among five additional patients in whom local control was not achieved, two developed pulmonary metastases 5 months after primary treatment. The remaining three have been free of pulmonary metastases for 8+–14+ months.

Weekly High-Dose MTX Treatment (Table 1)

During the past year four to eight weekly courses of VCR, MTX, and CF were administered to eight patients with pulmonary metastases or unresected primary lesions. The regimen is identical to that

[6]Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

TABLE 1.—Results of therapeutic regimens utilized in patients with osteogenic sarcoma

Treatment	No. of patients	Results	Comments
Adjuvant VCR, MTX, and CF (June 1972)*	12, local control	7, free of pulmonary metastases 2+–3 ½+ yrs	Metastases resected, 1 patient New tumor in contralateral nonamputated femur at termination of treatment, 1 patient Died of disease, 2 patients Calcified metastases, 1 patient (*see* below)
Adjuvant VCR, MTX, CF, and adriamycin (June 1974)*	20, local control	16, free of pulmonary metastases 6+–18+ mos	Pulmonary metastases resected in all 4 relapsed patients Inguinal and iliac nodes, 1 patient—resection followed by weekly VCR, MTX, CF, and radiation therapy (*see* below) Metastases in neck of femur and rib, 1 patient—resected
Weekly VCR, MTX, and CF (overt disease) (June 1974)*	4, pulmonary metastases (no prior MTX)	2, complete response 1, partial response 1, no response	Duration of complete response, 4–8+ mos
	4, primary tumor (no prior MTX)	3, complete response 1, partial response (*see* below)	
	5, pulmonary metastases (prior MTX)	2, partial response 3, no response	Duration of response, 2 mos
Weekly VCR, MTX, CF, and radiation therapy (post-surgical resection) (Nov 1974)*	4, pulmonary metastases		Tumor-free 3+–14+ mos
	1, inguinal and iliac nodes		Tumor-free 3+ mos
	1, inoperable osteogenic sarcoma of pelvis	50% reduction in tumor size with subjective relief	Duration of response, 8+ mos
Tri-weekly VCR, MTX, and CF (Jan 1971)*	14, pulmonary metastases (updated from original study [1])	2, complete response 2, partial response	Alive and well 5 yrs, 1 patient
	2, pulmonary metastases (treated after termination of original study)	2, calcification and stabilization of lesions	Anaphylactic reaction, 1 patient; duration of response, 1+ and 3¾+ yrs respectively

*Date study was started.

reported earlier except that escalations of MTX from 3000 to 7500 mg/m² are introduced at weekly intervals. The treatment produced two complete and one partial response in four patients with pulmonary metastases. The duration of response varied from 4 to 8+ months. Metastases which recurred in a patient with a complete response were removed surgically. Complete responses (disappearance of pain and radiographic improvement) were also obtained in three of four patients with primary lesions; these were removed surgically. In one patient with an extremity lesion, the response permitted a local en bloc resection of tumor with preservation of the limb by inserting a Buchholz total shoulder prosthesis. These patients had not received prior MTX treatment. In contrast, two partial responses were obtained among five patients with pulmonary metastases who had received prior VCR, MTX, and CF therapy; the duration of response was 2 months.

Radiation Therapy and VCR, MTX, and CF (Table 1)

Weekly VCR, MTX, and CF courses were administered in combination with radiation therapy to patients who relapsed on conventional MTX and adriamycin therapy. Four patients with residual tumor following surgical resection of pulmonary metastases were treated. The dose of radiation therapy was 1200–1600 rads administered in 150-rad frac-

tions to both whole-lung fields. One patient also received 1800 rads/9 FX/11 days (total 3000 rads in 32 days) to residual tumor located in the pleuropericardial area. These patients remain free of recurrent disease for 3+–14+ months.

One patient with residual disease in the pelvis following node resection was also treated. She received 5200 rads/27 FX/57 days. The treatment was interrupted after the second weekly VCR, MTX, and CF course because of toxicity and was resumed after a 2-week delay. She has remained free of recurrent disease for 3+ months. One additional patient received weekly VCR, MTX, and CF courses concurrently with radiation therapy (4000 rads/FX 20/28 days) for an inoperable osteogenic sarcoma of the pelvis. He had previously received 3000 rads to the tumor and adriamycin at another institution. A 50% reduction in tumor size was achieved with subjective response. A 4-mev accelerator was used in all patients.

Toxic Effects

There has been no alteration in the incidence of toxicity, as outlined in an earlier communication (6). The safety of MTX infusions has been improved with the availability of pharmacologic monitoring of serum MTX levels. Values in excess of 1×10^{-7} M at 72 hours are potentially toxic. The results call for extended CF administration and measures to improve hydration and MTX excretion. An elevated serum MTX level may also be associated with a "third space" (6).

An acute anaphylactic reaction occurred in one patient with pulmonary metastases while receiving an MTX infusion. This reaction was manifested as urticaria, moderate bronchospasm, and pruritus. She had tolerated previous tri-weekly VCR, MTX, and CF courses extremely well except for one severe episode of toxicity manifesting as myelosuppression, stomatitis, hepatitis, and oliguria. Upon recovery, three subsequent courses were also well tolerated until the anaphylactic reaction. Further treatment was declined. Of interest, however, is the appearance of calcification in the pulmonary metastases which have remained stable during the past year.

Pulmonary Metastases (Table 1)

Responses to weekly VCR, MTX, and CF in patients with pulmonary metastases were described earlier.

With the tri-weekly VCR, MTX, and CF schedule, two complete and two partial responses among 14 patients were obtained (17) (updated from original reports) (1,2). The duration of response varied from 2 months to 5+ years. The only surviving patient who remains free of disease has recently given birth to a live normal infant without evidence of congenital anomalies.

One patient with multiple small pulmonary metastases was treated with tri-weekly VCR, MTX, and CF for 3¼ years (and more recently adriamycin). Her metastases calcified and appear stable. Biopsy of the lesions demonstrated calcification and ossification with no evidence of viable tumor. She displays no evidence of acute or chronic MTX toxicity.

Discussion

The VCR, MTX, and CF program has had a major impact on the biologic behavior of osteogenic sarcoma. Its application as adjuvant treatment has reduced the incidence of pulmonary metastases. The latter, when they occurred, were characterized by a delayed onset and a decrease in number and size, rendering them amenable to surgical resection.

Stabilization of pulmonary metastases occurred in one patient for 12+ months following discontinuation of chemotherapy after anaphylaxis. Similar observations were noted in a patient treated with VCR, MTX, and CF over 3¼ years. Prolonged treatment of this nature is only justified if continued growth of metastases does not occur during its administration. VCR, MTX, and CF should, however, be supplanted if any other potentially effective therapeutic modality is available.

The development of a second bone tumor following discontinuation of adjuvant VCR, MTX, and CF treatment is of concern. The tumor could represent a new malignancy or a metastasis from the original lesion. Emergence of the tumor may also be related to extended survival in a susceptible host. Finally, the role of chemotherapy as a potential oncogen must also be considered. These factors indicate the importance of surveillance of long-term survivors.

Osteogenic sarcoma has been considered relatively radio-resistant. However, with the addition of VCR, MTX, and CF, an enhanced therapeutic response may be achieved (1). In contrast to reports by Rosen et al (8), this approach did not produce inordinate degrees of pulmonary fibrosis. Substantiation of these results will provide strong inducement for investigating the utility of radiation therapy and weekly VCR, MTX, and CF in prophylactic treatment of the lungs.

Superior results were achieved with weekly VCR, MTX, and CF in patients who had not received prior MTX therapy. Of particular importance was the response to treatment of the primary tumors. His-

torically, advances in the management of osteogenic sarcoma were limited because of concentration on methods to achieve local control of these lesions. The results suggest that this objective with destruction of pulmonary micrometastases may be achieved with the weekly schedule. Future investigation will be concentrated in this direction.

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High-Dose Methotrexate With Citrovorum Factor Rescue for the Treatment of Central Nervous System Tumors in Children [1,2]

Gerald Rosen,* Fereshteh Ghavimi, Anita Nirenberg, Crisanta Mosende, and Bipin M. Mehta [3,4]

SUMMARY

Eleven patients with brain tumors recurrent after surgery and radiation therapy were treated with high-dose methotrexate (MTX) (300–500 mg/kg) with citrovorum factor rescue (CFR). MTX was given as an iv infusion over 4 hours followed 2 hours later by oral CFR (10 mg every 6 hours × 12). MTX levels were measured in the serum, lumbar cerebrospinal fluid (CSF), and ventricular CSF at 0, 4, 12, 24, 48, and 72 hours from the start of the MTX infusion. MTX concentrations of $> 10^{-6}$ M were measured in the CSF over a period of 24 hours after the iv infusion. Peak CSF concentrations reached were $> 10^{-5}$ M. After the response to high-dose MTX with CFR was evaluated, vincristine (1.5 mg/m^2) and a nitrosourea (methyl-CCNU or BCNU) were added to this treatment regimen. Two of four patients with recurrent pontine glioma and five of seven patients with recurrent medulloblastoma had favorable objective responses to treatment. It is suggested that high-dose MTX with CFR be cautiously considered for the primary treatment of intracranial neoplasms in children with the hope of increasing the cure rate for children with malignant brain tumors.

[Cancer Treat Rep 61:681–690, 1977]

Since 1972, high-dose methotrexate (MTX) (200–500 mg/kg) with citrovorum factor rescue (CFR) has been used in the treatment of osteogenic sarcoma (1,2). We noted an increase in the survival of 15 patients with metastatic osteogenic sarcoma following treatment with high-dose MTX with CFR. Most of these patients had advanced metastatic disease and eventually died, although they had initial favorable responses to high-dose MTX with CFR (2). An autopsy performed on some of those patients who had received very large cumulative doses of high-dose MTX with CFR revealed normal histopathology of both brain and liver. The latter finding was somewhat surprising since it had been well documented that leukemic children and patients with psoriasis receiving far less MTX during their treatment developed fibrotic changes in the liver secondary to multiple-dose MTX administration (3,4). An analysis of the brain and liver tissue obtained at autopsy from patients with metastatic osteogenic sarcoma who had died after receiving many treatments with high-dose MTX and CFR revealed not only the accumulation of MTX in these tissues but an equal, if not greater, accumulation of CF.[5] This latter finding, which was associated with normal hepatic and normal cerebral tissue histology, led us to speculate that perhaps the administration of the CF following the MTX had protected the liver from the damage that can be caused by the multiple-dose administration of MTX. This led us to further speculate that perhaps CF could protect a normal brain from the damaging effects of MTX administration that had been reported when systemic MTX was given following brain irradiation (5), and perhaps

[1] Supported by Public Health Service grant CA-05826 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-. Supplied by the NCI, Bethesda, Md.

Citrovorum factor: NSC-3590; CAS reg. No. 6055–86–5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt. Supplied by Lederle Laboratories, Pearl River, NY.

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[4] We wish to acknowledge our appreciation to Mrs. J. Fusaro for her assistance in the preparation of this manuscript.

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[5] Rosen G, and Mehta BM. Unpublished data.

allow us to achieve a differential or a therapeutic effect on neoplastic tissue situated within the brain parenchyma.

In addition, the finding of the accumulation of MTX in the brain tissue made it obvious to us that high-dose MTX administered through the iv route was indeed crossing the blood-brain barrier. With this background, we decided to examine the role of high-dose MTX with CFR as a phase I agent in the treatment of recurrent intracranial neoplasms in children. A gratifying response was obtained in the first child treated with high-dose MTX with CFR after the recurrence of a pontine glioma following radiation therapy. This patient was treated for > 6 months with high-dose MTX (500 mg/kg) and CFR. The patient had an objective tumor response, as documented by serial pneumoencephalograms, that lasted for approximately 6 months, after which time the tumor recurred and the patient died. We had previously reported this case (6) prior to the patient's death. At autopsy, an infiltrating glioma of the pons was found. The tumor was a mixed astrocytoma containing predominant elements of glioblastoma multiforme. The favorable response of this patient prompted us to extend our studies with high-dose MTX with CFR in the treatment of intracranial neoplasms in children.

A protocol for the treatment of children with recurrent brain tumors was designed to allow us to evaluate high-dose MTX with CFR as a single agent initially. After the initial evaluation of high-dose MTX with CFR as a single agent, vincristine and BCNU[6] are added to the treatment protocol, since both of these agents have been shown to be effective in the treatment of brain tumors in children (7–9) (fig 1).

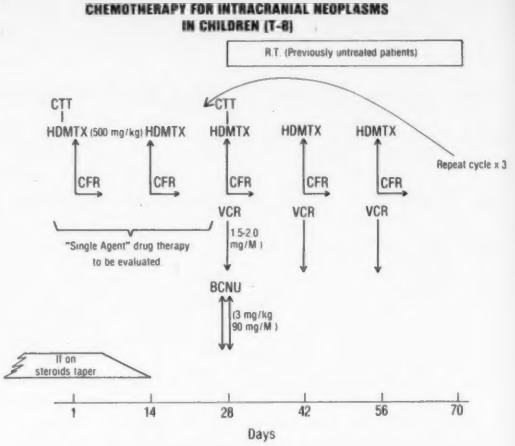

FIGURE 1.—Chemotherapy protocol for patients with intracranial neoplasms. Following the evaluation of the initial response to high-dose MTX with CFR, vincristine and BCNU are added to the treatment protocol. Patients receive a total of 14 doses of high-dose MTX with CFR given every 2 wks and 4 courses of BCNU. Following the completion of chemotherapy, patients are followed with repeat CTT scans with iv contrast every 2 mos.

METHODS

Eleven children with recurrent intracranial neoplasms following radiation therapy were entered into the treatment protocol. Seven patients had recurrent medulloblastoma and four had recurrent pontine glioma. The time of recurrence and the start of chemotherapy ranged from ½ to 61 months following the completion of initial radiation therapy. The characteristics of the patient population are enumerated in tables 1 and 2. The seven patients with recurrent medulloblastoma all had an initial surgical biopsy of their tumor. One patient's primary tumor had arisen in the cerebral hemisphere and the other six with recurrent medulloblastoma had their primary tumor initially in the cerebellum. All patients had received initial cranial and spinal irradiation, with maximum doses given to the posterior fossa or primary tumor-bearing area, of from 4000 to 6000 rads. Of the four patients with pontine gliomas, all had roentgenographic evidence of a pontine tumor. One patient had been previously reported and at autopsy was found to have a solid infiltrating tumor of the pons consisting primarily of glioblastoma multiforme (patient 8) (6). Two patients had evidence of a solid tumor on computerized transaxial tomography (CTT) of the brain following the administration of iv contrast. The fourth patient with a pontine tumor had only initial pneumoencephalographic evidence of recurrent tumor following radiation therapy. This patient (patient 10) failed to respond to high-dose MTX with CFR therapy.

Prior to therapy, all patients were evaluated for objective evidence of tumor recurrence either by pneumoencephalography or CTT scan with iv contrast. Following neurologic evaluation and evaluation of the patient's general status, renal function was assessed with a creatinine clearance. Patients with evidence of increased intracranial pressure were evaluated for placement of an intraventricular shunt, or revision of an existing non-functioning

[6] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1). Supplied by Eli Lilly and Co, Indianapolis, Ind.
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

TABLE 1.—High-dose MTX with CFR in combination chemotherapy for patients with recurrent medulloblastoma

Patient No.	Age/ sex	Previous radiation therapy (rads) (cranial/spinal/tumor*)	Site of recurrence	Response to chemotherapy	No. of high-dose MTX doses/ duration of response (mos)	Comments
1	10/F	4000/4000/4000 (15 mos from radiation therapy to high-dose MTX)	Multiple intracranial nodules	Decrease in size of all nodules on 2nd CTT scan of the head; no steroids	12/11+	Off chemotherapy for 5+ mos with no evidence of disease; no steroids
2	12/M	4000/4000/4000 No response of recurrent tumor to BCNU and methyl-CCNU 6 mos prior to high-dose MTX (61 mos from radiation therapy to high-dose MTX)	Frontal lobe tumor and spinal metastases	Disappearance of hypothalmic and frontal lobe tumors on CTT scan; neurologic improvement; steroids tapered over 3 mos	9/9+	Chemotherapy stopped (due to thrombocytopenia); upon recovery, partially necrotic tumor was excised
3	5/M	3700/3150/5700 Intrathecal Au^{198} (10 mos from radiation therapy to high-dose MTX)	Posterior fossa and spinal metastases	Disappearance of intracranial tumor on CTT scan; marked neurologic improvement; steroids tapered over 3 mos	12/7	Received additional 600 rads to posterior fossa; tumor recurred 3 mos after stopping chemotherapy; died of disease
4	3/M	4000/3600/5000 (3 mos from radiation therapy to high-dose MTX)	Multiple intracranial nodules	>50% reduction in size of all nodules on CTT scan; steroids stopped after 4 wks	11/6	Persistent subdural effusion after shunt; died at 6 mos, no autopsy; probable subdural hemorrhage during thrombocytopenia following chemotherapy
5	7/F	4500/2200/6000 (½ mo from radiation therapy to high-dose MTX)	Thoracolumbar spine, positive CSF cytology	Alive 34+ mos from start of high-dose MTX; last CSF cytology negative; no evidence of disease	12/34+	Developed degenerative encephalopathy 6 mos from start of high-dose MTX and 6½ mos from completion of 6000 rads to mid-cerebral fossa
6	12/M	5160/3300/5160 (8 mos from radiation therapy to high-dose MTX)	Cervical and thoracic spine and frontal lobe	Progressive disease	13/0	Concomitant radiation therapy to spine and orbit; died of disease 12 mos after start of high-dose MTX
7	5/F	3500/4000/5000 Intrathecal Au^{198} (20 mos from radiation therapy to high-dose MTX)	Frontal lobe	Progressive disease	7/0	Concomitant radiation therapy to frontal lobe; died with progressive disease 11 mos after start of high-dose MTX

*Tumor site was the posterior fossa in all except patient 5 whose primary site was the temporal lobe.

TABLE 2.—High-dose MTX with CFR in combination chemotherapy for patients with recurrent pontine glioma

Patient No.	Age/ sex	Previous radiation therapy (rads) to posterior fossa	Site of recurrence	Response to chemotherapy	No. of high-dose MTX doses/ duration of response (mos)	Comments
8	9/M	4950 (5 mos from radiation therapy to high-dose MTX)	Posterior fossa	Shrinkage in size of pons; neurologic improvement; steroids stopped after 2 wks	11/6	Did not receive vincristine or nitrosourea; autopsy proved pontine glioma to be a mixed glioma with areas of glioblastoma multiforme
9	10/M	5000 (1 mo from radiation therapy to high-dose MTX)	Posterior fossa	Dramatic neurologic improvement; steroids tapered after 4 mos of unsupervised administration by mother	13/12	Stopped chemotherapy after 11 doses of high-dose MTX (14 mos); tumor recurred 4 mos later, resistant to further high-dose MTX (2 doses)
10	12/M	5600 (5 mos from radiation therapy to high-dose MTX)	Posterior fossa	No objective improvement; steroids tapered	4/0	Started treatment in very poor condition; required tracheostomy; died of *Pneumocystis carinii* pneumonia
11	9/M	4000 (5 mos from radiation therapy to high-dose MTX)	Posterior fossa	Progressive disease; remained steroid dependent	9/0	Died of disease 7 mos after start of chemotherapy

shunt which was performed prior to the beginning of chemotherapy. In one patient a shunt was placed following the initial response to high-dose MTX with CFR when his large ventricular size failed to improve following his response to therapy (patient 4). All patients were receiving steroid therapy at the time of referral, and an attempt to taper steroids was undertaken following the first dose of high-dose MTX with CFR therapy. Ideally, patients were scheduled to undergo repeat roentgenographic studies after two or three doses of high-dose MTX with CFR. The initial dose of high-dose MTX with CFR was 300 mg/kg of MTX infused over a period of 4 hours. This was followed 2 hours later by CFR, the CF being given at a dose of 10 mg orally every 6 hours for 12 doses. After the first treatment, subsequent doses of MTX were increased to 500 mg/kg. The method of administration of high-dose MTX with CFR has been previously described (2,10) as well as the necessary clinical and laboratory followup of patients required to minimize systemic toxicity (11,12). All patients were followed with daily serum creatinines and MTX determinations (13) for 3 days following the administration of high-dose MTX. In addition, in four of the first five patients treated, MTX levels were determined in the cerebrospinal fluid (CSF) following high-dose MTX administration. The latter was achieved by both lumbar puncture in all patients, and the aspiration of ventricular fluid via Ommaya reservoir that had previously been placed in two patients for the purpose of measuring MTX levels following treatment, and through existing shunt reservoirs in two other patients. Nine of the 11 patients were given vincristine and nitrosourea therapy along with high-dose MTX with CFR following the evaluation of the response to high-dose MTX with CFR. Four of the nine patients then received methyl-CCNU[7] (120 mg/m^2 orally) 2 hours prior to the administration of vincristine and high-dose MTX with CFR, and five of the nine patients were subsequently treated with BCNU. BCNU was substituted for methyl-CCNU because of the improved response to brain tumors reported with its use (14) and because of the reliability of iv drug administration. High-dose MTX with CFR treatments were given every 2 weeks when the patient's condition permitted. Vincristine was given

[7] Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

at a dose of 1.5 mg/m² iv 1 hour prior to the administration of high-dose MTX. BCNU was given every 6 weeks, or with every third course of vincristine and high-dose MTX with CFR. BCNU was given at a dose of 90 mg/m²/day iv for 2 days, the first dose being given on the day prior to high-dose MTX with CFR and the second dose being given on the following day just before the administration of vincristine (fig 1).

RESULTS

Distribution of MTX

The average values for MTX concentrations in the serum and CSF obtained from four patients in whom the study was done are shown in figure 2. The average serum and CSF MTX concentrations following the administration of 300 mg/kg of MTX did not differ significantly from those obtained following the administration of 500 mg/kg of MTX.

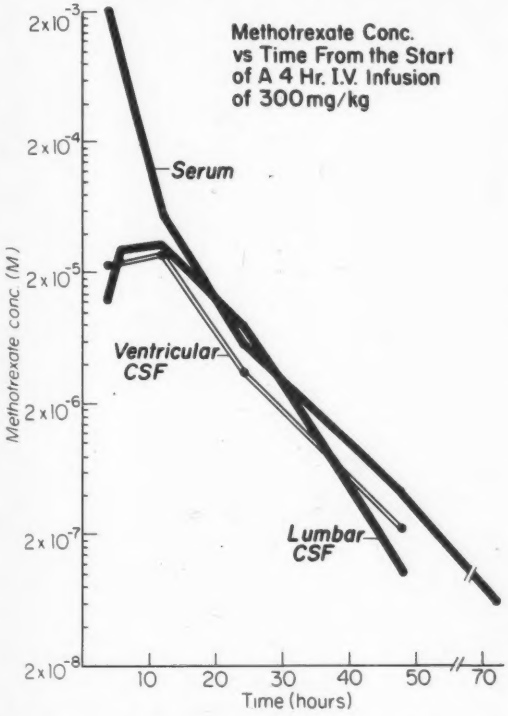

FIGURE 2.—Average MTX concentrations obtained from 4 patients following the administration of high-dose MTX (300 mg/kg). Serum, ventricular CSF, and lumbar CSF specimens were obtained at 0, 4, 12, 24, 48, and 72 hrs from the start of the high-dose MTX infusion.

Following the iv administration of high-dose MTX, there did not appear to be significant differences between the MTX concentrations in the ventricular CSF and those in the lumbar CSF. Peak MTX concentrations in the CSF were obtained 12 hours from the start of the 4-hour high-dose MTX infusion. Peak values were $> 10^{-5}$ M. MTX levels $> 10^{-7}$ M were recorded in the CSF for periods > 50 hours following the administration of high-dose MTX (fig 2). These levels are in excess of what has been reported to be cytotoxic levels of MTX for experimental tumor models in vitro (15,16).

Toxicity

The systemic toxicity of high-dose MTX with CFR has been well described (2,12). Toxicity was limited to moderate oropharyngeal mucositis in eight patients and severe mucositis in three. The three patients developing severe oropharyngeal mucositis following high-dose MTX (500 mg/kg) responded to local oral hygiene and mycostatin for the treatment of *Candida albicans* superinfection. Nitrosourea toxicity has been well described and consisted of anorexia, vomiting, and thrombocytopenia (17). Most patients experienced moderate thrombocytopenia 3–4 weeks following the third or fourth dose of either methyl-CCNU or BCNU.

One patient (patient 5, table 1) developed a degenerative encephalopathy 6 months from the start of the high-dose MTX with CFR therapy and 6½ months from the completion of 6000 rads to the mid-cerebral fossa. Neurologic deterioration began after her 12th dose of high-dose MTX with CFR. The syndrome was characterized by a slight elevation of the CSF protein, initial neurologic symptoms of mild ataxia, and weakness progressing to spastic quadriplegia, intellectual deterioration, and aphasia. A biopsy of the cerebral hemisphere revealed severe degenerative changes with reactive gliosis in the white matter. There was no evidence of viable tumor in the biopsied material, although a biopsy was not done at the site of the original primary tumor. It was felt that this "leukoencephalopathy" could be attributed to radiation therapy (the patient received a total of 6000 rads to the mid-cerebral fossa and a total of 4500 rads to the rest of the whole brain), or to the combination of radiation therapy and chemotherapy with high-dose MTX with CFR. The patient has remained in a vegetative state including spastic quadriplegia and aphasia for the past 28 months. Her last spinal tap done 21 months ago failed to reveal the presence of malignant cells on cytologic examination, and the CSF protein was 50 mg/100 ml. There has been no further progression of neurologic deterioration or evidence of recurrent medulloblas-

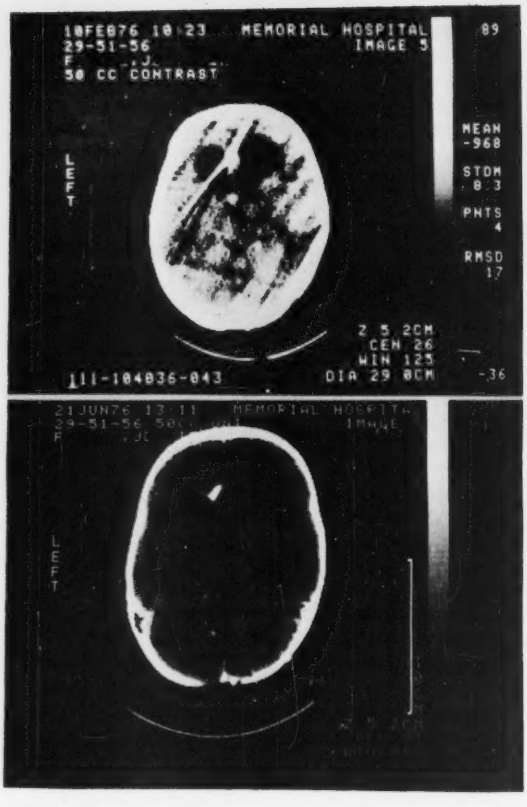

FIGURE 3.—Patient 1 (table 1): CTT scan with iv contrast prior to high-dose MTX with CFR chemotherapy shows multiple contrast-enhancing areas of recurrent tumor (top). 4 mos following the start of chemotherapy, repeat CTT scan shows a disappearance of all contrast-enhancing areas. The intraventricular shunt catheter can be seen in place, and the ventricles are still enlarged, probably due to prior long-standing obstruction (bottom). This patient continues to do well 5 mos following the cessation of all chemotherapy.

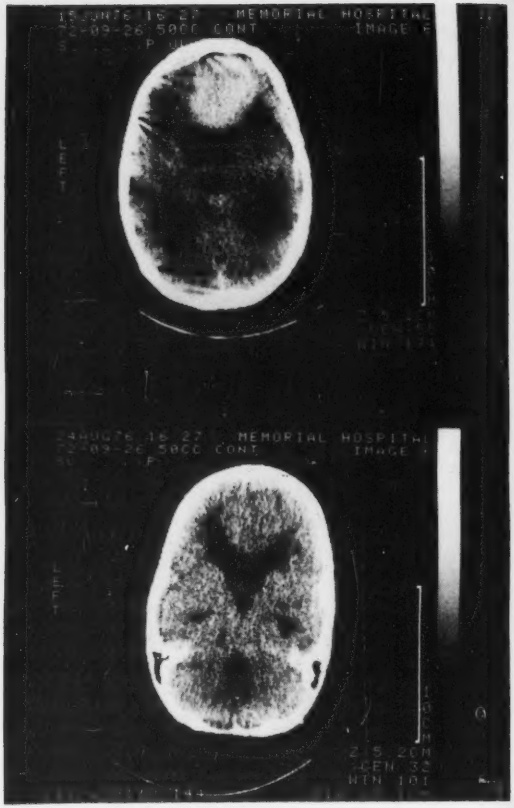

FIGURE 4.—Patient 2 (table 1): CTT scan demonstrating large recurrent medulloblastoma in the frontal lobe (top). Following chemotherapy with high-dose MTX and CFR, there is marked reduction in contrast enhancement in that area (bottom). Clinical improvement in the patient's condition paralleled the improvement in the CTT scan. This patient was treated with BCNU and methyl-CCNU prior to being referred for high-dose MTX with CFR. His treatment was started in the face of existing thrombocytopenia. After obtaining the above response to high-dose MTX with CFR, chemotherapy was stopped due to persistent severe thrombocytopenia. The patient witnessed a reappearance of contrast enhancement in the frontal lobe area and subsequently underwent surgical excision. At that time, it was noted that a large portion of the tumor was necrotic. Following surgery, the patient was again given high-dose MTX with CFR and continues to be in clinical remission.

toma. However, there has also been no improvement. The patient is cared for at home by her mother.

One patient (patient 4, table 1) had a ventriculoperitoneal shunt inserted because of persistent large ventricles after CTT evidence of disappearance of tumor was noted following initial chemotherapy. After the shunt procedure the patient developed bilateral subdural effusions. Five months later, the patient appeared to deteriorate clinically with the symptoms of lethargy, focal seizures, and breathing difficulty. This condition was further aggravated by electrolyte imbalances as a result of vomiting and poor fluid intake. It was postulated that some of his symptoms might be secondary to an increase in his bilateral subdural effusions. The patient died 12 days after chemotherapy with high-dose MTX with CFR, vincristine, and BCNU. This was his third dose of BCNU (fig 1) on the chemotherapy protocol, and at the time of his death he had thrombocytopenia secondary to chemotherapy.

Permission for autopsy was not obtained; however, it is likely that this patient's death was secondary to subdural hemorrhage due to thrombocytopenia following chemotherapy.

Results of Treatment

Table 1 enumerates seven patients with recurrent medulloblastoma who were treated with high-dose MTX with CFR. All patients had previously received radiation therapy to the entire neuroaxis. Radiation therapy is denoted as: total dose to the cranial vault/the total dose to the spinal axis/and the total maximum dose delivered to the tumor site. The site of the primary tumor was the posterior fossa in all patients except patient 5 who had a primitive neuroectodermal tumor of the temporal lobe which was interpreted as compatible with medulloblastoma arising in that area. The median time from the completion of radiation therapy to the beginning of chemotherapy was 10 months with a range of ½–61 months. Six months prior to being referred for high-dose MTX with CFR therapy, one patient had received methyl-CCNU and BCNU in an unsuccessful attempt to treat recurrent tumor.

Four of the seven patients had objective evidence of tumor regression as determined by serial CTT scan of the head performed with iv contrast prior to high-dose MTX with CFR treatment and after two to three treatments with high-dose MTX with CFR. In three of the four patients, the initial response continued to the point where all evidence of contrast-enhancing density disappeared on the CTT scan (figs 3 and 4). One patient (patient 4) had a > 50% reduction in the size of all nodules on the CTT scan following two doses of high-dose MTX with CFR. One patient (patient 5) with recurrent disease, including positive CSF cytology, was started on high-dose MTX with CFR therapy only ½ month following the completion of radiation therapy which was protracted over a 6-month period. Six months later, she developed a degenerative encephalopathy as described above and was taken off all therapy. She is alive without evidence of disease at 34 months from the start of chemotherapy; however, she remains in a vegetative state as noted above. Two of the seven patients with recurrent medulloblastoma had no response to high-dose MTX with CFR therapy. It should be noted that both of these patients had concomitant radiation therapy given to the area of recurrent disease at the time of starting high-dose MTX with CFR. The other patients who had responded to chemotherapy did not have additional radiation therapy while chemotherapy was being evaluated. One patient (patient 1) has completed all chemotherapy (14 doses of high-dose MTX with CFR and four doses of BCNU, fig 3), has been off all therapy for 5+ months, and continues to have no evidence of disease. However, the initial hydrocephalus that was present with the recurrent tumor has not resolved and the large ventricular size is probably due to brain atrophy as a result of previous long-standing obstruction to CSF pathways by tumor (fig 3). The median duration of response in the five patients with recurrent medulloblastoma was 9 months with a range of 6–34+ months. The response rate of recurrent medulloblastoma to high-dose MTX with CFR in this small series of patients is five of seven or 71%.

The results of treating four patients with recurrent pontine glioma are shown in table 2. All four patients were started on high-dose MTX with CFR from 1 to 5 months after the completion of radiation therapy to the posterior fossa. All patients experienced only brief responses to radiation therapy and had rapidly recurring symptoms following the completion of radiation therapy. Two of the four patients had objective improvement following high-dose MTX with CFR. The first patient (patient 8) was treated only with high-dose MTX with CFR and his case report was the subject of an earlier paper (6). This patient had a response to chemotherapy documented by serial pneumoencephalography. The response lasted for 6 months, at which time he had symptoms of recurrent tumor and was placed on other therapy. The patient eventually died from his disease and at autopsy his pontine tumor was found to be a massive mixed glioma with predominant elements of glioblastoma multiforme. Of interest is the fact that he had tumor seeding of the entire neuraxis down to the cauda equina which had not produced symptoms during his life. Another patient (patient 9) had rapidly deteriorating symptoms 1 month following the completion of 5000 rads to the posterior fossa. He had dramatic neurologic improvement following a protracted recovery from severe mucositis due to his third dose of high-dose MTX with CFR. Prior to treatment, he was unable to walk, could barely talk with a very nasal voice, and could not swallow. Following the response to high-dose MTX with CFR, he was able to walk, feed himself, swallow without regurgitation, and talk quite normally. However, he still had residual neurologic signs of cranial nerve palsies including a persistent sixth-nerve palsy. He continued to do well and after 11 doses of high-dose MTX with CFR (14 months) chemotherapy was stopped. Four months following the cessation of chemotherapy, neurologic evidence of tumor recurrence developed, and after he failed to respond to an additional two doses of high-dose MTX with CFR, he died of progressive disease at home. Two additional patients with recur-

rent posterior fossa pontine glioma had no objective improvement following high-dose MTX with CFR. Of the two patients with pontine glioma responding to therapy, one responded for 6 months and one responded for 12. Both patients had their steroid dose tapered and discontinued during their initial response to chemotherapy.

DISCUSSION

The distribution of MTX in the CSF following iv high-dose MTX with CFR demonstrates cytostatic levels of MTX in the CSF for > 48 hours (15,16), with peak levels being reached 12 hours after the beginning of the 4-hour iv infusion of MTX. The half-life of MTX in the CSF then closely followed that of MTX in the serum. The significance of these levels of MTX in the CSF in the treatment of intracranial neoplasms is uncertain. It would be of interest to measure concomitant folinic acid levels following CFR to determine if during the period immediately following the systemic administration of high-dose MTX with CFR, MTX levels in the CSF were capable of inhibiting incorporation of ^{3}H-deoxyuridine into DNA, since our preliminary data indicated that CF is accumulated in brain tissue over many months of treatment with high-dose MTX with CFR. However, whether or not a cytostatic effect on neoplastic tissue in the CSF is obtained for the first few days following high-dose MTX with CFR has not been determined.

We originally reported on the efficacy of high-dose MTX with CFR in the treatment of a patient with pontine glioma (6). Our enthusiasm for high-dose MTX with CFR in brain tumors was somewhat dampened by the experience of patient 5 (table 1) who developed severe degenerative encephalopathy 6 months after starting high-dose MTX with CFR. At this time, it had been reported that leukoencephalopathy developed following the iv administration of MTX to leukemic children who had previous cranial spinal irradiation (5). Although this patient has had no recurrent tumor and remains alive almost 3 years from the beginning of her treatment, we were highly suspicious of the fact that high-dose MTX with CFR could enhance the effect of previous irradiation to cause leukoencephalopathy in patients receiving this high-dose MTX with CFR. It is well known that MTX is capable of enhancing the effect of irradiation (18) and indeed, in our experience in treating osteogenic sarcoma, high-dose MTX with CFR delivered after completion of radiation therapy caused skin flare in the radiation therapy port, with an apparent enhancement of irradiation effect on normal tissue as well as upon the tumor (19). However, as initially stated, our preliminary data in examining brain tissue from patients with osteogenic sarcoma who had received numerous doses of high-dose MTX with CFR, including a patient who had received > 700 g of MTX in a year's time following the completion of radiation therapy for skull metastases of osteogenic sarcoma, revealed that none of these patients had developed leukoencephalopathy or any apparent permanent neurologic deficit after receiving high-dose MTX with CFR. Indeed, patient 5 (table 1) had received 6000 rads to the mid-cerebral fossa (only ½ month prior to starting high-dose MTX with CFR) and completion of radiation therapy in this patient was in the closest proximity to the beginning of high-dose MTX treatment of all our patients treated. In addition, the total cumulative dose given to this patient (6000 rads) was the highest dose of all patients treated and was in excess of what is usually delivered to the cranial vault or cranial spinal axis for intracranial neoplastic disease. Although the delayed effects of radiation therapy are usually most evident as vascular changes, there has been documentation in the literature of severe degenerative demyelinating disease accompanied by reactive gliosis in patients following radiation therapy alone. In 1959, Lampert et al (20) described a patient with disseminated demyelination of the brain occurring approximately 3 months after the delivery of a similar dose of radiation therapy (5700 rads) for the treatment of a recurrent basal cell carcinoma in the middle ear. Clinically, Lampert's patient had an onset of symptoms and course similar to those of our patient (patient 5), with the exception that Lampert's patient died approximately 3 months after the onset of this syndrome.

Nevertheless, the continued referral of patients with recurrent intracranial tumors and the impressive efficacy in treating these brain tumors in children with high-dose MTX with CFR gave us the impetus to proceed cautiously with this form of treatment in this relatively poor-risk group of patients. After the initial response to high-dose MTX with CFR was evaluated with CTT scans, vincristine and nitrosourea therapy was added to the treatment protocol because of the reported efficacy of these agents in the treatment of intracranial neoplasms (7–9). The majority of patients initially received methyl-CCNU orally; however, we have changed our treatment protocol to include BCNU given iv (fig 1) because of the apparent greater efficacy of iv BCNU in the treatment of malignant gliomas.

The favorable responses noted after high-dose MTX with CFR in two patients with pontine glioma make it imperative that we try to increase our experience in this disease and try to develop more

effective therapy than radiation therapy alone, since practically all patients with this disease have a relatively short survival even following radiation therapy.

The high favorable response rate of recurrent medulloblastoma to high-dose MTX with CFR indicates that this treatment is potentially beneficial in increasing the cure rate of medulloblastoma if it could be incorporated as part of the primary treatment following the initial surgery for medulloblastoma. The use of high-dose cranial spinal radiation in medulloblastoma has yielded a finite but still low cure rate in this disease (21). Although nitrosoureas and vinca alkaloids have been shown to be effective in treating recurrent medulloblastoma, it would certainly be desirable to add as many useful agents as possible to the initial treatment of this disease with the hope of obtaining the highest possible cure rate. The combination of high-dose MTX with CFR and radiation therapy for the primary treatment of brain tumors might indeed be synergistic; however, extreme caution must still be exercised in combining these treatment modalities for primary brain tumors, since the ability to destroy normal brain tissue with combination therapy is a real possibility. However, poor 5- and 10-year survival rates in children with intracranial neoplasms certainly warrant such a trial. In this setting, it might be appropriate to lower the dose of radiation therapy delivered to the entire cranial-spinal axis to that which gives maximal tumor response, and not use the maximally tolerated dose of normal brain tissue. By combining lower doses of total cranial and spinal irradiation with high-dose MTX with CFR as the initial treatment for primary intracranial neoplasms, it might be possible to increase the cure rate for this common neoplastic disease in children. It is notable that of the seven patients with recurrent medulloblastoma, the two who did not respond had concomitant additional radiation therapy delivered to the tumor-bearing area, and those patients responding to high-dose MTX with CFR did not receive additional radiation therapy to the recurrent tumor site being evaluated. This leads to speculation that perhaps the proper timing of combined chemotherapy and radiation therapy should include high-dose MTX with CFR prior to aggressive radiation therapy. Optimal combined therapy for primary medulloblastoma might include high-dose MTX with CFR administered prior to radiation therapy, with radiation therapy given in tumoricidal doses but at doses lower than those commonly used to treat medulloblastoma at this time, since these doses are the maximally tolerated doses in the brain and in themselves can cause delayed untoward neurologic effects. It would be best to find a still effective but lower dose of radiation therapy that could act synergistically with the chemotherapy to produce more prolonged tumor control with less toxic side effects. Following radiation therapy, perhaps combination therapy with high-dose MTX and CFR and other agents can be used as adjuvant therapy to increase the poor survival in children with intracranial neoplasms.

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High-Dose Methotrexate-Citrovorum Factor Rescue in the Management of Brain Tumors [1,2]

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SUMMARY

During studies of high-dose methotrexate (MTX) in acute leukemia, measurable levels of the antifol were detected in the cerebrospinal fluid of children given 15–100 mg/kg of this agent in 4-hour infusions. Substantial concentrations of MTX (0.7–3.25 μg/ml of cerebrospinal fluid or 1.54–7.15 $\times$ 10^{-6} M) were found in patients 12–24 hours following iv administration of the drug. The cerebrospinal fluid levels of MTX tended to be higher in the presence of central nervous system pathology. In view of these observations, a child with grade 3 astrocytoma recurring after surgery and radiation therapy was treated with intracarotid infusions of 5–70 mg/kg of MTX administered over 2–4 hours. Marked neurologic improvement was observed. After 1 year of periodic infusions of MTX, usually without citrovorum factor (CF) rescue, complete recovery was accompanied by normaiization of the angiogram. This patient is alive and well 7 years later without further treatment. Three other children with advanced pontine (two patients) and third ventricle (one patient) glioma were treated with iv high-dose MTX and CF rescue as described previously for lung carcinoma and used for osteogenic sarcoma. All three responded initially with marked but temporary neurologic improvement. Subsequent symptomatic responses were obtained in two of the three patients with increased doses of MTX ($\leq$ 1 g/kg). These two patients died 4–5 months following discontinuation of treatment. The third patient failed to respond to 2.2 g/kg of MTX. An adult patient with parieto-occipital glioma recurring after surgery, radiation therapy, and CCNU, responded to MTX-CF with complete neurologic recovery. Although her angiographic studies are currently normal, the computerized transaxial tomography scan shows continued presence of a tumor lesion. Pulse high-dose MTX has consistent antitumor activity in brain gliomas. Optimal dosage schedules of MTX-CF for this tumor have not yet been determined.

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The use of high-dose pulse methotrexate (MTX), with or without citrovorum factor (CF) rescue, was developed in the course of studies in childhood acute leukemia (1), lymphosarcoma (2,3), and primary carcinoma of the lung (4,5). Early in these studies we observed detectable amounts of MTX in the cerebrospinal fluid (CSF) of patients given high doses of this drug iv. The limited responsiveness of brain tumors to chemotherapy is often attributed to the inability of most chemotherapeutic agents to penetrate beyond the so-called blood-brain barrier. Drugs in the nitrosourea group (BCNU and CCNU), known for their ability to cross the barrier, are therefore emphasized in studies and practice, although with limited success. The detection of MTX in the CSF of patients with acute leukemia given high-dose MTX prompted us to investigate this phenomenon in patients with brain tumors. Preliminary observations on the transport of MTX into the

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[2]Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: NSC-3590; CAS reg. No. 6055–86–5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

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CSF and its possible effect on brain malignancies are the subject of this report.

MATERIALS AND METHODS

MTX without preservatives, packaged in 500-mg vials, was supplied by the National Cancer Institute, Bethesda, Md. CF was supplied by the Lederle Laboratories, Pearl River, NY. MTX in each vial was dissolved in 10 ml of distilled water and transferred to a 250- or 500-ml bottle with the balance to the full capacity of the container being made up with 5% dextrose in water. MTX was infused iv or intra-arterially over 4–24 hours as indicated below. Part of the total dose (usually 30%) was given rapidly, volume permitting, and the remainder of the dose was infused at a constant rate over the predetermined total time of infusion. CF was given im, starting 2–12 hours after completion of the infusion of MTX.

The dosage schedule of MTX and of CF was varied according to the response of the tumor and the ability of the patient to tolerate the treatment. A preferred dosage schedule for the treatment of brain tumors was derived from the experience described below. It is used at present for the continuing study as follows:

Day 1. MTX (100 mg/kg of body weight) given in 6 hours with 35 mg/kg of the total dose infused in 20 minutes at the start of the infusion followed 12 hours later by CF (0.2 mg/kg im) every 6 hours for eight doses.

Day 14. MTX (200 mg/kg of body weight) given in 6 hours with 70 mg/kg of the dose infused in 30 minutes at the start of the infusion followed 12 hours later by CF (0.3 mg/kg im) every 6 hours for ten doses.

Day 35. MTX (300 mg/kg) given as above in 6 hours followed 12 hours later by CF (0.3 mg/kg im) for 12 doses.

Day 56. MTX (600 mg/kg) given in a 6-hour infusion with 200 mg/kg of the total dose infused over 1 hour followed 12 hours later by CF (0.4 mg/kg im) given every 6 hours for 12 doses.

Day 77. MTX (900 mg/kg) given in 12 hours with 300 mg/kg of the total dose infused in 2 hours and followed by the remaining amount in a 10-hour infusion. Twelve hours later CF (0.4 mg/kg im) is given every 6 hours for 12 doses.

The last dose schedule is continued every 3 weeks. Creatinine clearance and liver function are determined prior to each treatment.

REMARKS

(*a*) The gradual escalation of the MTX dose can be halted at any of the levels described above if stable and adequate neurologic improvement occurs. Further escalation is resumed at first sign of clinical deterioration.

(*b*) A kidney function study by the creatinine clearance test prior to each treatment is mandatory. When values are below lower limits of normal therapy is postponed until their return to normal range (usually 7–10 days).

(*c*) Adequate hydration of the patient is ensured during and for at least 2 days after the administration of MTX by infusion of iv fluids sufficient to account for invisible water loss and a minimum of 500–1000 ml of urine per day.

(*d*) Insertion of a subclavian catheter is recommended for all patients, and especially for children, in order to ensure easy, immediate, and permanent access to the venous circulation. This catheter can be left in place after filling it with heparin and capping it.

(*e*) Beginning oral toxicity (salivation, redness of the oral mucosa, or mucosal ulcerations) within 76 hours (3 days) following MTX is an indication for continuing CF injections at 0.4 mg/kg every 6 hours for four to six additional doses. Skin rash, diffuse or perifollicular, is also an indication for additional CF.

(*f*) Upper respiratory infection or other viral infections are an indication for postponing treatments for 4–7 days.

(*g*) A CF-containing cream (1 mg/g of lanolin) can be applied on the lips or perirectally if signs of induration or ulceration are detected. CF solution (3 mg/10 ml of water) can be used for mouth rinsing every 2 hours if mouth toxicity develops.

(*h*) Mouth washes with Mycostatin (1 ml) and a solution containing nonadsorbable antibiotics (polymyxin, neomycin, and bacitracin) are recommended every 3 hours as described previously (1).

(*i*) Availability of platelets and possibly of granulocyte transfusions is mandatory.

(*j*) In-patient observation for at least 4 days after each treatment is recommended. Daily examination and blood counts on an out-patient basis for 3 more days are desirable.

(*k*) Blood levels of MTX are determined daily, starting 24 hours after infusion of the drug.

(*l*) Persistence of > 100 mμg of MTX/ml of serum after the last dose of CF is an indication for continuing CF rescue with at least four more doses of CF or until the MTX level (determined daily) is < 100 mμg/ml of serum.

TABLE 1.—MTX levels in CSF in patients with glioma after iv high-dose MTX

Patient No.	Iv MTX dose (mg/kg)	Serum MTX (μg/ml)	MTX in CSF (μg/ml)	Time after iv MTX (hrs)
1	100	50	0.75	3
	100	44	0.70	3
	150	80	1.2	3
2	300	136	3.25	2
3	500	—	1.0	9

TABLE 2.—Response of advanced and progressing brain tumors to high-dose MTX-CF rescue*

Patient No.	Age (yrs)	Tumor	Maximal single MTX dose (mg/kg)	Neurologic improvement	Survival time from—	
					Diagnosis (mos)	MTX therapy (mos)
1	11	Astrocytoma, grade 3	70, intracarotid 150, iv	Yes (+++)	119+(10 yrs)	109+
2	6	Glioma, brain stem	1200, iv	Yes (++)	23†	13
3	5	Glioma, brain stem	1200, iv	Yes (+)	15†	11
4	3	Ependymoma	1600, iv	Yes (+)	12	7
5	11	Astrocytoma	2200, iv	Yes (++)	22	8
6	51	Glioblastoma multiforme	400, iv	Yes (++)	7+	6+
7	21	Medulloblastoma	1000, iv	No (−)	17	2

*All patients failed to respond or were relapsing following radiotherapy. Initial subtotal resection was done in patients 1, 5, 6, and 7.

†Treatment stopped 4 and 5 mos prior to death.

The Werkheiser enzymatic technique (6) was used in our studies for quantitative determination of MTX levels in blood and CSF.

RESULTS

CSF levels of MTX were determined in four patients with acute lymphocytic leukemia in complete hematologic remission after iv infusion of 100 mg/kg of MTX in 4 hours. Two of these patients had no central nervous system (CNS) leukemia. Three hours later their levels of MTX in the CSF were 0.27 and 0.47 μg/ml respectively. The remaining two patients who had CNS leukemia showed two and three times higher levels of MTX in their CSF. Another patient (patient 5) with acute leukemia and leukoencephalopathy showed 12.3 μg/ml of fluid 3½ hours after receiving a dose of 200 mg/kg of MTX iv. No effect on his CSF cell count was observed. An autopsy confirmed the diagnosis of leukoencephalopathy without CNS leukemia.

The CSF levels of MTX following systemic administration of the drug were also determined in three patients with brain gliomas as shown in table 1. In these patients the MTX levels were higher than in the individuals with acute leukemia without CNS pathology. Higher CSF levels were observed when giving higher systemic doses of MTX. The levels at 3 hours after iv administration of 100, 150, and 300 mg/kg of MTX were increased in direct proportion to the dose of MTX infused.

A therapeutic trial with high-dose MTX-CF rescue in seven patients with brain tumors is summarized in table 2. Five of these patients had gliomas, one had ependymoma, and one had medulloblastoma. Only one patient (patient 1) received intracarotid perfusions prior to the usual iv treatments. Arterial perfusion was not possible in the remaining six patients. Objective neurologic improvement was observed in all except the patient with medulloblastoma. The improvement was usually greater after the first two or three treatments when relatively smaller doses of MTX were given (100–300 mg/kg). A second or third response, after neurologic relapse, was observed in four patients (patients 2–5) given higher doses of MTX. These responses, however, were of lesser degree and of shorter duration. The duration of the effects of 1200 mg/kg in patients 2 and 3 could not be evaluated since treatment was subsequently stopped in these two patients. The

neurologic impairment in patient 7 (medulloblastoma) progressed despite treatments with 100–1000 mg/kg of MTX. It recurred and eventually failed to respond in patients 4 and 5.

The survival of the five patients who died (patients 2–5 and 7) varied from 12 to 23 months. The three deaths in the glioma patients occurred at 15, 22, and 23 months after diagnosis. Progression of tumor growth despite MTX treatments, however, was documented at autopsy in only one of these three patients (patient 5). Two glioma patients are alive at 7+ months and 10+ years respectively. The second has not been receiving treatment for the last 8 years. The patient with ependymoma, despite excellent initial response, eventually progressed and died. A large and viable tumor was found at autopsy 1 month after a dose of 1600 mg/kg of MTX.

Moderate to severe toxicity, including mouth ulceration, skin rash, and bone marrow depression, occurred in all except patient 6. The toxicity in patient 1 occurred only after the second treatment, which consisted of 70 mg/kg of MTX given intra-arterially (via the right internal carotid artery) without CF rescue. Substantial transfusions of platelets and granulocyte concentrates were used in patients 5 and 7. Clinically significant impairment of renal function occurred in patient 7, although temporary reduction of creatinine clearance was occasionally observed in all others. No deaths due to MTX toxicity occurred in this group of patients.

DISCUSSION

The finding of measurable concentrations of MTX in the CSF of patients given high-dose MTX is of theoretic as well as practical interest. The nature of the blood-brain barrier is poorly understood. With regard to MTX at least, this barrier appears to be less than absolute. The finding of about 0.7 μg/ml of CSF for each 100 mg/kg of MTX given iv as shown in table 1 suggests that a percentage of this drug had crossed into the CSF. The existence of a "threshold" level in the blood, below which no passage occurs, cannot be excluded on this basis alone, although if it exists, this level must be rather low. It is of interest that the levels of MTX in the CSF were higher in patients with CNS pathology (brain tumors, CNS leukemia, and leukoencephalopathy) than in patients with acute leukemia in complete remission. This could be due to impaired efficiency of the barrier at the site of a tumor or degenerative process.

The responsiveness of some brain tumors to high-dose MTX is not surprising in view of previous reports of responses to intrathecal (7) or intralesional (8) use of MTX. In fact, Rosen et al (9) recently reported experience with high-dose MTX-CF quite similar to our observations. The outcome with patient 1, who is alive and well 8 years after stopping all treatment, is of course provocative, although by no means conclusive. This patient was reclassified histologically from the cells in the aspirated tumor fluid upon recurrences as having a grade 3 astrocytoma, despite initial histologic classification as grade 2. It should be noted that this patient was the only recipient of arterial perfusions with MTX. Neurologic improvement, nevertheless, occurred consistently in all five patients with gliomas. Although two patients are still alive and doing well (patients 1 and 6), three have died (patients 2, 3, and 5). Despite the consistent initial responses in the group as a whole (six of seven patients), decreased effectiveness requiring larger doses of MTX was eventually noted in four of the six responders.

The value of high-dose MTX-CF rescue in the management of brain malignancies needs further study. Trials of different dosage schedules of MTX and CF rescue in patients with early disease are indeed warranted.

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Weekly Methotrexate-Calcium Leucovorin Rescue: Effect of Alkalinization on Nephrotoxicity; Pharmacokinetics in the CNS; and Use in CNS Non-Hodgkin's Lymphoma [1,2]

Susan W. Pitman* and Emil Frei III [3]

SUMMARY

Urinary alkalinization with oral sodium bicarbonate has decreased the incidence of acute nephrotoxicity and subsequent myelotoxicity in 18 adults receiving high-dose methotrexate with calcium leucovorin rescue (MTX-LCV) weekly in doses of 1–7.5 g/m². Close monitoring of 24-hour serum creatinine and MTX levels can predict patients at risk for serious toxicity. By a prompt (24–36 hours) increase in the LCV dose rate, hematologic and biochemical evidence of myelosuppression has been prevented. Kinetic parameters in serum and lumbar cerebrospinal fluid (CSF) were studied in two patients following iv injection of 3 and 7.5 g/m² respectively. Lumbar CSF MTX concentrations > 1 μM are achieved. The half-life of MTX in the CSF (11.95 hours) is twice as long as the serum half-life. In the presence of carcinomatous meningitis, further delay in the clearance of MTX from the CSF was seen. With weekly MTX-LCV, there have been four objective responses in six patients with non-Hodgkin's lymphoma in CNS relapse, including complete regression in two. It is suggested that therapeutic concentrations can be achieved in the central nervous system following MTX-LCV.

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Acute nephrotoxicity has been recently recognized as a significant complication of high-dose methotrexate-calcium leucovorin rescue (MTX-LCV) administration in adults (1). In a recent study, 60% of adults had evidence of diminished renal function, manifest either as a > 50% rise in serum creatinine or acute nonoliguric renal failure. Since MTX is excreted primarily by the kidneys, in the presence of acute renal damage due to the drug itself, prolongation of the serum half-life results in inadequate rescue by standard doses of LCV with subsequent marrow and gastrointestinal toxicity (1,2). Because the solubility of MTX, a weak dicarboxylic acid, is reduced at least tenfold in an acid milieu, we postulated that the pathogenesis of this MTX-induced nephrotoxic renal failure might be intratubular precipitation of MTX, when urine pH was ≤ 5.7 (1). The following indirect evidence supported this hypothesis: (*a*) prompt elevation of serum creatinine within 24 hours after MTX; (*b*) yellow fluorescent amorphous tubular casts in urinary sediment within 2 hours after MTX; and (*c*) the presence of intratubular amorphous yellow material on an alcohol-fixed autopsied kidney. In the present communication, we report preliminary results of a trial of urinary alkalinization, begun in an attempt to decrease the incidence of nephrotoxicity associated with MTX-LCV administration. A number of parallel observations were made with respect to the pharmacology of MTX in the central nervous system (CNS) in an attempt to correlate therapeutic effect and toxicity with concurrent MTX levels in serum and cerebrospinal fluid (CSF).

[1]Supported in part by Public Health Service grant CA-06516 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Calcium leucovorin: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

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PATIENTS AND METHODS

Patients.—Eighteen adults with biopsy-proven neoplastic disease were studied. Their ages ranged from 20 to 68 years (median, 44 years). Written informed consent was obtained. Normal renal function, as evidenced by a normal intravenous pyelogram and a creatinine clearance > 60 ml/minute, was required. All patients except one had wbc counts > 3000/mm^3 and platelet counts > 100,000/mm^3. Catheters were placed into the lumbar subarachnoid space in two patients with carcinomatous meningitis and squamous cell carcinoma metastatic to the base of the brain. Concurrent CSF and blood samples from each patient were obtained from 0.5 to 24 hours after iv MTX injection.

Drug administration.—MTX was administered as a single iv push. The dose was 1–7.5 g/m^2 (table 1). Of the 18 patients, 12 were begun at and subsequently continued at a dose of 1 g/m^2. Of the remaining six patients, two were begun at 1 g/m^2 and escalated to 7.5 g/m^2 in an attempt to improve disease control, while four others began at 2 or 3 g/m^2 and were subsequently escalated. LCV was given 24 hours after MTX. The first dose of 10 mg/m^2 was given iv followed by 10 mg/m^2 orally every 6 hours for 12 doses. MTX-LCV treatments were repeated at 1-week intervals, usually in the outpatient department.

Urinary alkalinization.—Twelve hours prior to MTX administration, sodium bicarbonate, 3 g orally every 3 hours, was begun and continued for 48 hours following MTX administration. Patients were instructed to maintain urine pH > 7, using Combistix testing, and to maintain adequate oral hydration (> 3 liters of fluid/day). While waiting for the results of a stat creatinine, obtained immediately prior to each weekly course and then compared to the baseline value (prior to the first course of MTX-LCV) to assess adequacy of renal function, 1 liter of fluid was administered iv. If at 24 hours after MTX, the serum creatinine was > 50% above the baseline serum creatinine value, patients were instructed to increase oral hydration and to return at 36 hours for repeat serum creatinine determination. In all instances except one, serum creatinine values returned toward baseline. If, however, serum creatinine continued to rise at 36 hours, LCV was empirically increased to 100 mg orally every 3 hours until serum MTX concentrations returned to values < 1 × 10^{-7} M. Preservative-free MTX and LCV were dissolved in sterile water for injection before iv administration.

Determination of MTX.—Serum and CSF were assayed for MTX using the methods described by Bertino and Fisher (3), and more recently by radioimmunoassay, with comparable results in the range of 10^{-7} to 10^{-8} M (4). The dispositional rate constants were calculated by least-squares regression analysis of the individual serum MTX concentrations. These values were used to calculate the dimensions of a two-compartment open model (5). The CSF clearance was calculated using the formula Ce (ml/hour) = 0.7 Vd/T ½, assuming a CSF volume of 140 ml (6).

Evaluation of renal function.— The effect of a course of MTX-LCV rescue on renal function was considered evaluable if serum creatinine was determined before and during the week after the administration of MTX. In subsequent courses, serum creatinine levels during the week after each MTX course were compared with serum creatinine levels before the first course. A change in serum creatinine > 50% above the baseline was considered a significant change in renal function since the

TABLE 1.—MTX dose distribution

No. of patients	18
No. of courses at dose of—	
1 g/m^2	89
2 g/m^2	28
3 g/m^2	20
7.5 g/m^2	17
	154
No. of courses per patient	
Median	5
Range	1–29
Initial dose (g/m^2)	
Median	1
Range	1–3
Highest dose (g/m^2)	
Median	1
Range	1–7.5

TABLE 2.—MTX-LCV course distribution of patients who received 1 g/m^2 in all courses

Total No. of courses	No. of patients	Course No. with >50% serum creatinine elevation
1	1	
2	1	Course 2
3	4	
4		
5		
6	2	Course 1
.		
.		
.		
17	1	Courses 14, 16
.		
.		
.		
29	1	Course 13

TABLE 3.—Renal toxicity: frequency

	No. evaluable	Increase in serum creatinine		
		25%–50%	>50%	Total
Per course				
Non-alkalinized patients	73 courses	13(18%)	21(29%)	34(47%)
Alkalinized patients	154 courses	17(11%)	6(4%)	23(15%)
Per patient				
Non-alkalinized patients	33 patients	5(13%)	19(60%)	24(73%)
Alkalinized patients	18 patients	7(39%)	5(28%)	7(39%)

serum creatinine determination is reproducible in our laboratory (1 SD = 3.6%) and since multiple repetitive daily samples in normal stable subjects yield an SD of 12%.

Evaluation of myelosuppression.—Myelotoxicity was defined as a wbc count < 2000/mm³ and/or a platelet count < 100,000/mm³.

RESULTS

Nephrotoxicity

A total of six episodes of elevation of serum creatinine of > 50% occurred in the 154 evaluable courses, five at a dose of 1 g/m² (table 2) and one at 7.5 g/m². Table 3 shows the incidence of nephrotoxicity in this alkalinized group compared to an earlier group of 33 non-alkalinized patients who received 73 courses of MTX-LCV given tri-weekly (1). The age ranges and median ages of the two groups were comparable, as were entrance criteria for the study, except for an intravenous pyelogram requirement in the present study. It is evident that urinary alkalinization has decreased the overall incidence of nephrotoxicity (> 50% elevation of serum creatinine) from 29% to 4% of courses. Whereas 19 of 33 (60%) non-alkalinized patients exhibited an increase of ≥ 50% in the serum creatinine, only five of 18 (28%) alkalinized patients did. Since treatments in adults were rarely accompanied by nausea and vomiting, it is unlikely that dehydration accounted for the elevations in serum creatinine seen.

Table 4 compares the incidence of nephrotoxicity following the first course of MTX-LCV in the alkalinized weekly group and the non-alkalinized tri-weekly group. A statistically significant decrease in the incidence of serum creatinine elevations was seen, both at the 1-g/m² level and for all doses administered when the urine pH was maintained at ≥ 7 prior to and for 48 hours following MTX. Overall, 4.3% (five of 117) of courses following 1 or 2 g/m² and 2.7% (one of 37) following 3 or 7.5 g/m² were associated with increases of serum creatinine > 50%. It is probable that doses > 1 g/m² are above the threshold level of MTX required for renal injury, since two recent episodes of acute nephrotoxicity encountered at 80 and 160 mg/m² iv have been documented by us.

Myelotoxicity

Myelosuppression occurred in patients developing nephrotoxicity (20% of the non-alkalinized and 0.3% [two of 154] of the alkalinized courses). The two episodes of myelosuppression encountered in this alkalinized study were mild, neither requiring wbc or platelet support or hospitalization.

Use of the 24-Hour Serum MTX Determination

Figure 1 portrays the frequency distribution of serum MTX levels obtained in 52 courses, 24 hours after 1 g/m² of MTX by iv push. The median MTX level was 5.2×10^{-7} M. It is evident that MTX levels $< 1 \times 10^{-6}$ M are associated with no change in renal function and no toxicity. In contrast, MTX values $> 1 \times 10^{-6}$ M may be associated with a 24-hour elevation of serum creatinine > 50% and may predict myelotoxicity. By close monitoring of 24-hour serum creatinine and serum MTX determinations, prediction of patients at risk for toxicity has been possible. Toxicity has been aborted by prompt increase in the frequency, amount, and duration of LCV administration.

Case Report 1.—After administration of 1 g/m² of MTX by iv push the serum MTX at 24 hours was 5.5×10^{-6} M (5.5 μM), and the 24-hour serum creatinine was 3.0 mg/100 ml, the latter representing a 300% increase over the pretreatment serum creatinine (1.0 mg/100 ml) 24 hours earlier. The serum MTX also represented a log increase over a corresponding level of 4.8×10^{-7} M (0.48 μM) obtained 24 hours after the first course of drug. Severe myelotoxicity and mucositis were considered imminent because of (*a*) > 50% increase in serum creatinine and (*b*) greater than tenfold increase in plasma MTX compared to an earlier nontoxic course. LCV was empirically increased at 36 hours to 100 mg/m² iv every 3 hours and continued for the next 14 days until serum MTX levels had decreased to 4×10^{-8} M. Clinically, neither myelosuppression nor mucositis

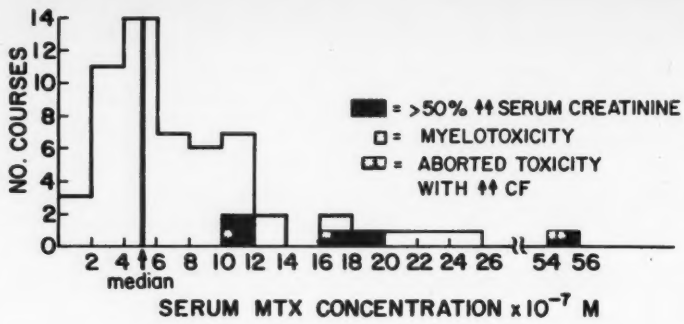

FIGURE 1.—Serum MTX concentrations at 24 hrs after 1 g/m² by iv push.

TABLE 4.—Renal toxicity after 1st course of MTX-LCV*

Patients	Dose (g/m²)	No. of patients	No. of courses with increase in serum creatinine of— ≤ 50%	≥ 50%
Alkalinized	1	13	12	1
	>1	5	5	0
	(3, median)	18	17	1
Non-alkalinized	1	5	1	4
	>1	28	18	10
	(2, median)	33	19	14

*Comparison of alkalinized and non-alkalinized patients at 1 g/m², $\chi_1^2 = 9.41$ $P < 0.01$; all doses, $\chi_1^2 = 7.61$ $P < 0.01$.

was observed. No reduction in [³H]deoxyuridine incorporation into trichloroacetic acid-precipitable DNA of bone marrow was seen and flow cytofluorometric histograms of DNA per cell showed no accumulation of cells in G_1–S.

Pharmacokinetics in the CNS

The kinetic parameters in serum and lumbar CSF following a single iv injection of 3 and 7.5 g/m² MTX are shown in table 5. Following 3 g/m², CSF concentrations of 10^{-5} M (10 μM) were achieved within 1 hour and maintained for at least 18 hours, at which time the catheter was removed from the lumbar subarachnoid space. The CSF half-life is prolonged (43.3 hours) in the presence of carcinomatous meningitis. Figure 2 shows that within 1 hour of a 7.5-g/m² iv injection, MTX concentrations in excess of 10^{-5} M (10 μM) are achieved in the CSF. The half-life of MTX in the CSF (11.95 hours) is twice as long as the serum half-life (5.6 hours).

CSF MTX levels were obtained 24 hours following systemic iv push administration of 3 g/m² in seven patients. The geometric mean CSF MTX concentration was 3.31×10^{-6} M. Following 7.5 g/m² in nine patients, the geometric mean CSF MTX concentration was 4.5×10^{-6} M. All CSF MTX levels, except one, were > 1 μM at 24 hours following 3 or 7.5 g/m² iv push.

TABLE 5.—Kinetic parameters in serum and CSF following single iv administration of 3 and 7.5 g/m² of MTX in 2 patients

Parameter	Dose 3 g/m²*	7.5 g/m²
V_1† (liter/m²)	6.1	7.35
Vdss‡ (liter/m²)	19.0	25.4
Clearance (ml/min/m²) §	70	52.5
Half-life α (mins)§	7	21
Half-life β (hrs)§	3.2	5.6
Half-life CSF (hrs)	43.3	11.95
CSF clearance (ml/hr)	2.26	8.2

*Carcinomatous meningitis.
†Central compartment volume.
‡Steady state distribution volume.
§Serum.

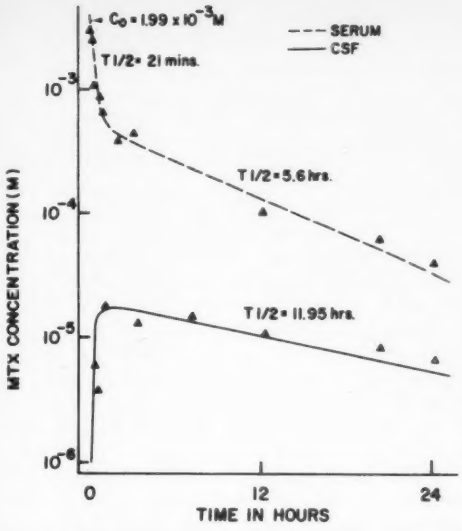

FIGURE 2.—MTX concentrations in serum and lumbar CSF after a single iv injection of 7.5 g/m².

CNS Tumor Response

Table 6 documents the responses of six patients with non-Hodgkin's lymphoma who had relapsed in the CNS following systemic therapy. Objective disease regression, of both systemic and CNS lymphoma, was achieved in four of six patients, with complete regression of all abnormalities in two. Malignant cells were identified in the CSF in four of six patients, and CSF protein was elevated in all five patients in whom it was measured. Patient 2 relapsed at 2 months when weekly MTX-LCV treatments were changed to every 3 weeks. A prompt partial response of intracranial lymphoma, easily measured on brain and CAT scan, was documented in patient 3 after 1 g/m², but relapse occurred at 6 weeks. With subsequent increase in the MTX-LCV dose to 3 g/m², followed by 7.5 g/m², a second regression was achieved which lasted 2 months. The response of patient 4 consisted of complete clearing of CSF malignant lymphoblasts with a decrease in CSF protein, but not to < 40 mg/100 ml. Patient 1 is illustrative of a durable response in lymphomatous meningitis.

Case Report 2.—A 28-year-old white man (patient 1 in table 6) in previously excellent health presented in October 1974 with dyspnea on exertion. A right pleural effusion and a right mid-zone infiltrate were present on chest radiograph. Thoracotomy revealed diffuse poorly differentiated lymphocytic lymphoma. Bone marrow biopsy was negative. Physical examination revealed no peripheral lymphadenopathy. Systemic chemotherapy was begun with bleomycin, adriamycin, cyclophosphamide, vincristine, and prednisone. A complete remission was achieved, and after 6 months of chemotherapy bacillus Calmette-Guérin scarifications were started and continued monthly. Eleven months after initial diagnosis, in October 1975, painful paresthesias of the right fifth cranial nerve, diplopia, and dysphagia were noted. Brain scan was negative. Lumbar puncture disclosed an opening pressure of 400 mm Hg, CSF protein of 222 mg/100 ml, and a wbc count of 60/mm³, all malignant lymphocytes. There was anterior and lateral displacement of the left ureter, with new left periaortic lymphadenopathy on intravenous pyelogram. MTX at a dose of 2 g/m² by iv push was begun. CNS symptoms cleared after three weekly courses. After nine weekly courses at 2 g/m² examination of the CSF with cytocentrifuge prep revealed no evidence of malignant lymphocytes with 1 wbc/mm³. The 24-hour CSF MTX levels were 8.8×10^{-7} M

TABLE 6.—MTX-LCV therapy of CNS non-Hodgkin's lymphoma

Patient No.	Histology*	Age (yrs)/ sex	Clinical signs and symptoms	CSF cytology	CSF protein (mg/100 ml)	CNS response (mos)	Survival after MTX-LCV (mos)
1	DPDL	28/M	Headache, cranial nerve palsy	+	222	CR (14+)	15+
2	DH	54/M	Headache, CAT scan positive	+	1000	CR (2)	2.5
3	DPDL	34/M	Headache, seizure, CAT and brain scans positive	Not determined	Not determined	PR (4)	4.5
4	DPDL	68/M	Headache, leg paraplegia	+	168	PR (1)	3.5
5	DH	33/M	Headache, leg paresis, cranial nerve palsy	—	165	No response	3
6	DPDL	55/M	Headache, back pain	+	150	No response	11

*DPDL = diffuse poorly differentiated lymphoma; DH = diffuse histiocytic.
†CR = complete response (disappearance of all measurable disease); PR = partial response (>50% reduction in measurable disease).

(concurrent serum level, 2.8×10^{-6} M) and 8×10^{-7} M (serum, 1×10^{-6} M). Neither steroids nor irradiation was given. Intravenous pyelogram returned to normal. In November 1975, when complete remission was documented, a consolidation program including MTX-LCV, cyclophosphamide, vincristine, and prednisone every 3 weeks was begun. Duration of this complete response has been 14+ months.

DISCUSSION

The results presented suggest that maintenance of urinary pH ≥ 7, if begun 12 hours before and continued for 48 hours after MTX, has decreased the incidence of nephrotoxicity following MTX-LCV administration in adults. The efficacy of urine alkalinization suggests that intratubular precipitation of drug must be contributing, at least in part, to the pathogenesis of the renal injury. Immunofluorescence of a recent renal biopsy, performed because of acute renal failure following MTX, has revealed, using fluorescein-labeled MTX antisera, highly fluorescent tubular casts and tubular basement membranes in the kidney, adding direct evidence for support of this hypothesis.[4]

A metabolite, 7-hydroxy-MTX, whose aqueous solubility is threefold to fivefold less than MTX (7), has been detected in urine of human subjects and rhesus monkeys receiving MTX. However, in this report, the excretion of the metabolite was < 10% of the total excreted drug in the early 6–12-hour human urine samples, while in our series, fluorescent crystalline urinary casts have been detected as early as 2 hours after MTX administration. It is possible, however, that 7-hydroxy-MTX may contribute to the formation of this crystalline intratubular material.

A reversible decline in glomerular filtration rate (GFR), as measured by inulin and creatinine clearances, has been observed in pediatric patients receiving high-dose MTX in the absence of serum creatinine changes.[5] If the mechanism of this mild renal injury is intratubular precipitation of drug, rather than an antifol effect, urinary alkalinization might be expected to abolish the decline in the GFR.

By close monitoring of 24-hour serum creatinine and serum MTX levels, early recognition of delayed MTX clearance has been possible. By predicting patients at risk of serious toxicity to bone marrow and intestinal epithelium and by empirically increasing the dose rate of LCV, we have been successful in aborting toxicity. Although this approach appears useful in the management of such patients, it is empiric and it may be that in the presence of very high MTX concentrations, LCV at any dose may be ineffective. Measurement of serum 5-methyl-tetrahydrofolate in the presence of MTX will be necessary before a less empiric approach in reversing MTX-induced inhibition of DNA synthesis is possible.

Concentrations of MTX capable of inhibiting DNA synthesis (8,9) were achieved in the CSF following iv injections of MTX-LCV in doses between 2 and 7.5 g/m^2. The objective responses seen in CNS non-Hodgkin's lymphoma suggest that these concentrations are therapeutic. Although Shapiro et al (10) found negligible ventricular concentrations following a single iv injection of MTX, the dose administered (500 mg/m^2) was much lower than that used in the present study. The duration of high CSF MTX concentrations following a single iv injection of MTX-LCV suggests that further therapeutic trials in leukemic and carcinomatous meningitides should be considered as well as its early use in tumors with a high CNS relapse rate, such as non-Hodgkin's lymphoma or breast adenocarcinoma, to achieve therapeutic concentrations in the CNS "sanctuary."

The half-life in the CSF following a single systemic injection of 7.5 g/m^2 of MTX-LCV is similar to that seen following intrathecal administration of 12–15 mg/m^2 of MTX (11). The prolonged half-life in this compartment as compared to the serum might be predicted since MTX, a weak dicarboxylic acid (pKa 4.7), would be largely ionized at the usual CSF pH of 7.4–7.6.

In the presence of carcinomatous meningitis there was further delay in the clearance of MTX from the CSF. It is possible that in the presence of malignant meningitides, transport of MTX from the CSF to the systemic circulation is impaired by the presence of malignant cells.

Neurotoxicity was not seen in this group of patients. However, since the neurotoxicity associated with intrathecal MTX treatment is more common in patients with meningeal leukemia, and since it may be related to prolonged exposure to MTX concentrations in the CSF (11), this side effect will have to be carefully monitored.

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Clinical Pharmacology of Intrathecal Methotrexate. I. Pharmacokinetics in Nontoxic Patients After Lumbar Injection [1,2]

Werner A. Bleyer [3,*] and Robert L. Dedrick [4]

SUMMARY

Cerebrospinal fluid (CSF) and plasma methotrexate (MTX) concentrations were measured in 76 children and adults who received intrathecal MTX, 12 mg/m² body surface area, without developing symptoms of lumbar puncture syndrome or drug-induced neurotoxicity. After an initial rapid redistribution of the injected drug, the mean antifolate value in the lumbar CSF declined in a biphasic manner with half-lives of 4.5 and 14 hours. In the plasma the mean MTX concentration reached a peak of 2×10^{-7} M between 3 and 12 hours after injection and decreased biexponentially thereafter with half-lives of 5.5 and 24 hours. Pharmacokinetic analysis suggested that the predominant mode of MTX transfer from the central nervous system to the systemic circulation is bulk flow absorption of CSF with entrained drug. The two phases of disappearance from the lumbar CSF appear to result from interaction of the convective transport and diffusion between the CSF and brain extracellular fluid (and perhaps some intracellular sites).

[Cancer Treat Rep 61:703–708, 1977]

The neurotoxicity of methotrexate (MTX) therapy has recently been correlated with the total amount of MTX administered (1–3) and with elevated concentrations of the drug in the cerebrospinal fluid (CSF) (4). Although there are several reported studies on the distribution kinetics of intrathecal MTX (4–7), the clinical pharmacology of this therapeutic modality and the factors affecting the disposition of MTX within the central nervous system (CNS) remain largely undefined. This report describes the CSF and plasma pharmacokinetics of MTX after lumbar injection in 76 nontoxic patients, and attempts to characterize those factors which determine the kinetics of distribution.

PATIENTS AND METHODS

CSF samples were obtained in 120 children and adults administered MTX via lumbar puncture for prophylaxis or treatment of meningeal leukemia. Forty-four patients had signs or symptoms related to overt meningeal leukemia, to lumbar puncture syndrome, or to intrathecal MTX neurotoxicity and will be described in a separate report. The remaining 76 patients received intrathecal MTX without developing therapy-related neurologic toxicity. This "asymptomatic" group of patients comprised the data base for this study.

The mean patient age was 11.3 years (range, 0.5–64 years) and the mean body surface area was 0.96 m² (range, 0.4–2.0 m²). In 71 patients, CSF samples were obtained only during lumbar puncture for a repeat dose of intrathecal chemotherapy. In five patients, serial lumbar punctures were performed to further characterize the disappearance kinetics. In 14 patients, serial plasma samples were also obtained after intrathecal injection.

All patients received MTX via lumbar puncture at a dose of 12 mg/m² diluted in Elliott's B solution to a concentration of 1.0–1.5 mg/ml. The volume of injection was 12 ml/m², with a maximum volume of 18 ml; it was injected slowly without barbotage after iso-

[1] Supported by Public Health Service grant CA-16525 from the National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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volumetric removal of CSF. MTX concentration was determined in triplicate by the dihydrofolate reductase inhibition method (8), with use of an enzyme derived from L1210 leukemic cells resistant to MTX (provided by Dr. David G. Johns, NCI). The assay has a lower limit of sensitivity of 4×10^{-10} M in CSF and 1×10^{-9} M in plasma. The only drugs known to interfere with the assay are other folate antagonists such as trimethoprim and pyrimethamine. Patients receiving these drugs, or given MTX systemically during the 3 days before study, were excluded.

The values for the various body parameters used in this study were derived as follows: for the average patient in this study (11 years old, 35 kg body weight, 0.96 m^2 body surface area), it was assumed that the CSF volume was 140 ml and that the combined weight of the brain and spinal cord was 1500 g (9,10). Assuming that the extracellular space (ECS) was 18%–20% (11), the extracellular fluid (ECF) volume of the brain and spinal cord was calculated to be 285 ml. From the sum of the CSF volume and the ECF volume of the brain and spinal cord, the total extracellular volume of the CNS was estimated to be 425 ml. For individual patients, the volume of the CSF and the weight of the brain and spinal cord were corrected for age (10) and the body parameters were recalculated as described above.

RESULTS

MTX redistributed rapidly from the injection site, with an initial half-disappearance time of < 1 hour (fig 1). The subsequent biphasic curve was characterized by half-lives of 4.5 and 14 hours for the intervals of 4–36 and 48–96 hours after injection, respectively (fig 1). The apparent volume of distribution calculated by extrapolation of the 4.5-hour half-life was 480 ml. This volume corresponded with a value of 425 ml for the volume of ECF within the CNS. The mean antifolate concentration in the CSF remained $> 10^{-7}$ M for 48 hours and did not decline to 10^{-8} M until 96 hours.

Since the above findings were derived from a composite of single determinations in individual patients, serial CSF samples were obtained in one patient—a 3-year-old boy with chronic meningeal leukemia. The ECF volume of his CNS was estimated at 350 ml. The second and third exponential phases were demonstrated (fig 1, inset) and their respective half-disappearance times were 5 and 19 hours. The apparent volume of distribution was 400 ml. As in the combined group of patients, this value approximated the patient's extracellular volume of the CNS.

To determine whether or not the kinetics of disappearance were concentration-dependent, the dosage was varied in some patients from 1 to 12 mg/m^2, and serial CSF samples were obtained. The half-life of the second phase of disappearance remained constant and the concentration was proportional to dose (fig 2), confirming first-order kinetics for this phase. First-order kinetics could not be conclusively demonstrated for the third phase of distribution, however. In some instances, the half-life of the third phase appeared to be directly proportional to the dosage.

MTX concentrations in the plasma reached a peak of 2×10^{-7} M between 3 and 12 hours after injection and then fell biexponentially with half-lives of 5.5 and 24 hours (fig 3). The plasma disappearance curve approximately paralleled the CSF disappearance curve. Similar findings were observed when serial plasma and CSF samples were obtained in one patient—a slender (53 kg, 192 cm, 1.65 m^2) 18-year-old boy (fig 3, inset).

DISCUSSION

This report describes the pharmacokinetics of intrathecal MTX in patients who were treated prophylactically and did not develop symptoms of lumbar puncture syndrome, chemical arachnoiditis, or neurologic dysfunction after drug administration. By excluding symptomatic patients, the effects of altered CSF flow, pressure, and protein content on the distribution kinetics should have been minimized. The results reported here, then, should represent the "normal" pharmacokinetics of intrathecal MTX therapy in asymptomatic patients under controlled clinical conditions. The interpretation of results is further simplified by the fact that MTX is not metabolized within the CNS.

Disappearance of MTX from the lumbar CSF reflects several different physiologic processes and anatomic compartments. The primary determinants seem to be (*a*) bulk flow distribution within the subarachnoid CSF (and to some extent within ventricular CSF); (*b*) diffusion into and through the ECS of the brain parenchyma and spinal cord; (*c*) back diffusion from the ECF of brain parenchyma into the CSF, and removal from the CNS by (*d*) bulk flow of entrained drug via the normal pathways of CSF absorption and by (*e*) diffusion from the ECF into the capillaries of the brain and spinal cord. Other processes may include (*f*) diffusion into the arachnoid and dural membranes and removal via the circulating blood of the meninges, (*g*) absorption from ventricular fluid by an energy-dependent transport mechanism located in the choroid plexus (14), and (*h*) uptake by certain cells in the brain and spinal cord.

Although we have not attempted to include all of

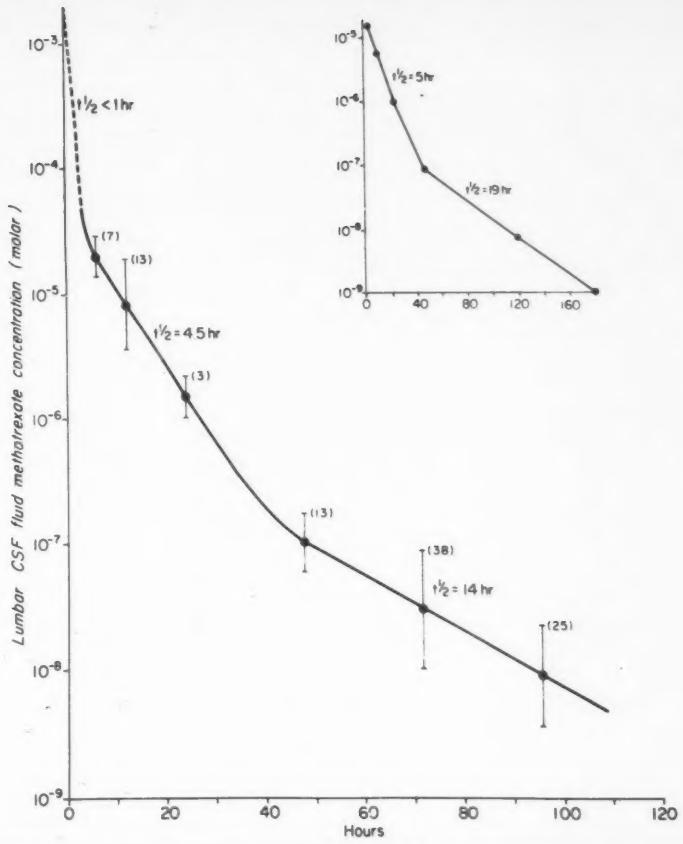

FIGURE 1.—Kinetics of MTX disappearance (geometric mean ± 1 SD) from lumbar CSF in 76 patients administered 12 mg/m². The concentration at time 0 is the concentration of MTX in the volume (6–18 ml) injected. Nos. in parentheses indicate the No. of patients studied. *Inset:* lumbar CSF-MTX concentration in 1 patient; initial redistribution phase is not depicted.

the above factors in a detailed, mathematic analysis of transport within the CNS, the magnitude of certain parameters can be assessed with the use of the simplified lumped model shown in figure 4. The model assumes that MTX distributes into a CNS space, V_{CNS}, equal to the volume of the CSF plus the volume of the ECF of the brain and spinal cord. This assumption is supported by the close correlation between the apparent volume of distribution and the extracellular volume of the CNS. It is also supported by the ventriculospinal perfusion studies of Rubin and associates (15), in which steady-state concentrations of MTX and inulin were identical. The model also assumes that drug transport from the CNS to the systemic circulation occurs during bulk flow absorption of CSF (Q_{CSF}), by passive transport (PA), and by active transport processes (r). The mass balance equation expressing these processes is given in figure 4, with r = rate of transport by active processes, PA = (permeability) (area) product for diffusion between blood and CSF, Q_{CSF} = rate of CSF formation, Cp = plasma concentration, and C_{CSF} = CSF concentration. Anatomic considerations suggest that choroidal transport is unlikely to significantly alter disappearance from the lumbar subarachnoid space. Hence, if it is assumed that there is no significant active transport of MTX from CNS (ie, r = 0), then at steady state ($d C_{CSF}/dt = 0$) the mass balance equation can be reduced to

$$(PA)(C_{CSF} - Cp) + Q_{CSF} C_{CSF} = 0$$

and rearranged to

$$PA = Q_{CSF}\left(\frac{C_{CSF}}{C_P - C_{CSF}}\right).$$

From Davson (9), Q_{CSF} = 31.2 ml/hour. From Shapiro et al (5) and Dedrick et al (13), $C_{CSF}/C_P \cong 30$ at steady state. Thus,

$$PA \cong 31.2\left(\frac{1}{30-1}\right) = 1.07 \text{ ml/hour.}$$

The value of PA calculated above is only 3% of the normal CSF flow rate. Under the assumption of negligible active transport of MTX between blood and the CSF, then, bulk flow clearly dominates the removal.

Since $PA << Q_{CSF}$ and $C_P << C_{CSF}$ under the conditions of this study, the mass balance equation shows a first-order elimination with a rate constant of

$$\frac{PA + Q_{CSF}}{V_{CSS}} = \frac{1 + 31.2}{425} = 0.076 \text{ hour}^{-1}.$$

The predicted half-life for MTX clearance, then, is 0.693 ÷ 0.076 = 9.0 hours. This half-life approximates the average of the observed values of 4.5 and 14 hours for the two principal phases of the disappearance curve. It is qualitatively consistent with the suggestion above that MTX removal from CSF is influenced first by diffusion into the brain parenchyma and subsequently by diffusion back into CSF after the net concentration gradient changes direction. Poorly mixed recesses within the subarachnoid space may also return drug to the CSF. Back diffusion would be expected to require many hours. This mechanism is in qualitative agreement with the change in the slope of the disappearance curve 40 hours after the injection.

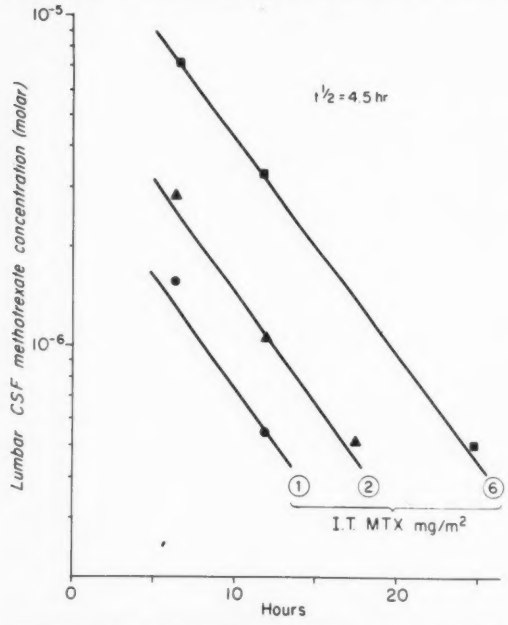

FIGURE 2.—Lumbar CSF-MTX concentrations in 1 patient after injections of 1, 2, and 6 mg/m². The solid lines for 1 and 2 mg/m² were predicted from the curve for 6 mg/m², assuming first-order kinetics.

None of the above considerations can exclude active transport at the choroid plexus or extrachoroidal sites. Nor can they absolutely exclude some binding or uptake by certain cells of the CNS. From the above analysis, however, these do not appear to be dominant factors in the distribution kinetics of intralumbar MTX. We would also conclude that the initial rapid redistribution phase probably reflects bulk flow of CSF within the subarachnoid space, that the second phase probably includes diffusion into the ECS of the CNS, and that the third phase may represent a combination of back diffusion and the processes which mediate the removal of MTX from the CNS.

Caution should be exercised in the application of any lumped model such as shown in figure 4 to predict the distribution of MTX and its effect on neoplastic cells within the CNS. It is known, for example, that drug concentrations vary from site to site within the CSF (5). The concentration profile within the parenchyma of the CNS may also differ significantly from region to region. In addition, the presence of tumor masses or tissue adhesions may alter the dynamics of bulk flow and diffusion such that the malignant cells are not exposed to effective drug concentrations even though the lumbar concentration-time history would be expected to be therapeutic.

Delineation of the clinical pharmacology in nontoxic patients should now permit evaluation of how the kinetics are altered in patients with neurotoxic reactions. It should also be possible to elucidate those factors which modify the disposition of MTX in the CNS and which may alter its efficacy and toxicity in man. Hopefully, the concepts derived will help improve the therapeutic index of intrathecal MTX and of intrathecal chemotherapy in general.

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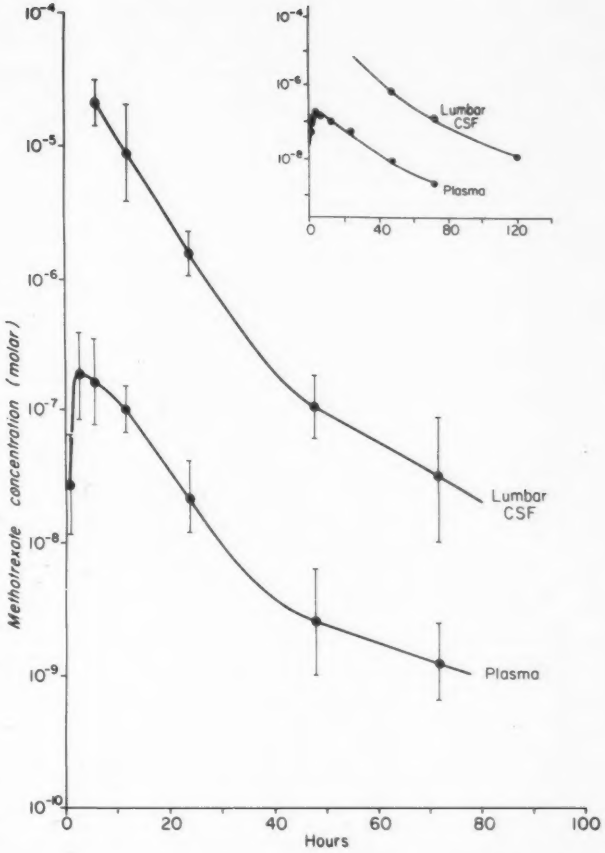
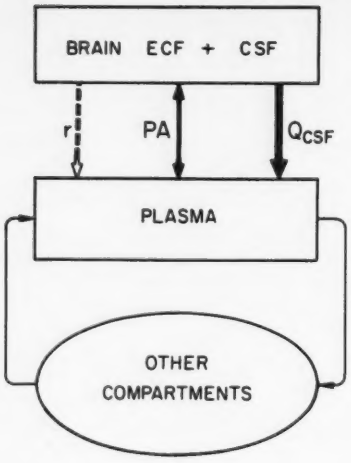


FIGURE 3.—Plasma MTX concentrations (geometric mean ± 1 SD) in 14 patients treated with 12 mg/m² compared with the lumbar CSF-MTX values depicted in fig 1. *Inset:* plasma and lumbar CSF-MTX concentrations in 1 patient.



$$-V_{CNS}\frac{dC_{CSF}}{dt} = r + (PA)(C_{CSF} - C_P) + Q_{CSF}\,C_{CSF}$$

FIGURE 4.—Compartmental model depicting transport processes between the CNS and the systemic compartments (adapted from Gabelnick et al [ref 12] and Dedrick et al [ref 13]). *See* text for definitions of abbreviations.

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Role of Drug Concentration, Duration of Exposure, and Endogenous Metabolites in Determining Methotrexate Cytotoxicity[1]

Herbert M. Pinedo[2] and Bruce A. Chabner[3,*]

SUMMARY

The cytotoxic effects of methotrexate for normal and neoplastic tissues are well known to clinicians but the scheduling of drug administration has primarily been based on empiric observation. However, experimental studies in recent years have provided a rational basis for understanding the role of specific drug concentrations, durations of exposure, and physiologic environment in determining the cytotoxicity of methotrexate. The studies reviewed in this paper demonstrate the requirement for free drug in excess of approximately 10^{-8} M concentration for inhibiting DNA synthesis in mouse bone marrow in vivo and in vitro, and in various cell culture lines in vitro. Rapid reversal of this inhibition is possible by removal of free drug by washing of cells, enzymatic cleavage of drug, or removal of infusion source in vivo. This requirement for free drug is thought to be the result of the incomplete titration of dihydrofolate reductase by stoichiometric amounts of drug, as demonstrable in in vitro competitive binding experiments. Further studies with constant drug infusion in mice have delineated the relative importance of duration of exposure and drug concentration in determining bone marrow toxicity. It was shown that for drug concentrations $> 10^{-8}$ M, the rate of depletion of nucleated bone marrow cells was directly related to plasma methotrexate concentration and reached its nadir of 30% of control within 12 hours at 10^{-5} M MTX, 24 hours at 2×10^{-6} M MTX, and 48 hours at 2×10^{-7} M MTX. The rate of loss of nucleated cells was directly related to duration of exposure until the nadir in cell count was reached. Depletion of total nucleated cells was mitigated somewhat by probable recruitment of previously uncommitted precursor cells to myeloid colony-forming potential during the second 24 hours of drug infusion. It was also found that 10% fetal calf serum as well as thymidine (10^{-5} M) plus a purine nucleoside could prevent the cytotoxic effect of methotrexate on bone marrow colony formation in vitro, indicating that endogenous nucleosides may be important in determining cell-kill.

[Cancer Treat Rep 61:709–715, 1977]

The primary working relationship for clinical pharmacology is the correlation between plasma-drug concentration and biologic effect. Thus, the readily available assays for diverse drugs such as digitalis, glycosides, antibiotics, and others have made it possible to establish concentration ranges for safe and therapeutically effective use of these agents. Quantitative abnormalities in the renal or hepatic clearance of these and other drugs may be detected by assay of serum concentrations and the dose can be adjusted to accommodate these changes. In the use of antineoplastic drugs, such assays have not been generally available, and with the possible exception of methotrexate (1,2), cytosine arabinoside (3), and adriamycin (4), drug schedules at present are based on empiric observation using

[1] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

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gross parameters such as organ toxicity and clinical antitumor effect as the major guidelines. The only clear exception to this clouded picture is the developing knowledge of the clinical pharmacology of methotrexate and, specifically, the relationship between cytotoxicity and the concentration of free drug in plasma (5) and cerebrospinal fluid (6). It will be the purpose of this paper to summarize recent work which has established (*a*) the requirement for free drug in excess of the tightly bound fraction for inhibition of DNA synthesis; (*b*) the threshold concentration of free drug for inhibition of DNA synthesis in target organs; (*c*) the relationship of drug level and duration of exposure to cytotoxicity at concentrations above the threshold; and (*d*) the possible influence of endogenous factors such as competitive substrate, thymidine, and purine nucleosides on the cytotoxic effects of methotrexate on mouse bone marrow. Although very little of this work has been performed in man, the correlations observed undoubtedly hold true in a qualitative sense, and with the development of improved assay techniques and renewed interest in methotrexate (7,8), confirmation of these findings in man is to be expected in the near future.

The Role of Free Methotrexate

Methotrexate is a strong inhibitor of bacterial and mammalian dihydrofolate reductases, displaying stoichiometric binding to the mammalian enzyme under conditions of slightly acid pH (6.1) and limited competitive substrate (9). The K_i for this inhibition has not been precisely determined by conventional enzyme-kinetic methods because of technical problems associated with measuring enzyme rates under the conditions required, although estimates in the range of 10^{-10} to 10^{-11} M have been made (10). Using competitive protein-binding methods, Myers et al (8) were able to determine a K_i of 5×10^{-9} M for the *Lactobacillus casei* reductase, and a similar figure for enzyme from the human lymphoblastoid line, CEM.[1] In these competitive binding experiments, as well as in conventional enzyme kinetics, it was apparent that in order to completely saturate the ligand protein, a considerable excess of free drug was required (fig 1). In considering the more alkaline pH of the intracellular milieu and the increase in competitive substrate, dihydrofolate (11), which takes place following blockade of reductase, it would be expected that a considerable excess of "free" methotrexate would be required to saturate the enzyme.

[1]Myers CE. Personal communication.

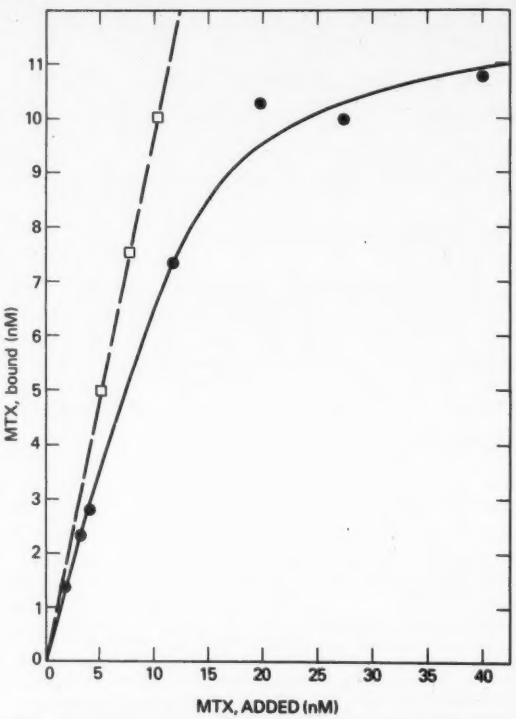

FIGURE 1.—Titration curve of methotrexate (MTX) concentration vs drug bound at pH 6.2, 0.17 mM phosphate buffer with 0.53 mM nicotinamide adenine dinucleotide, reduced form, at 37°C. The volume of incubation is 1 ml. Dihydrofolate reductase capable of binding 15 pmols of MTX was also present. The dotted line represents the theoretic, stoichiometric titration of 1 mol of MTX per mol of reductase, while the solid line represents the observed titration curve.

These theoretic considerations correlate well with the observed requirements for methotrexate cytotoxicity in cell culture systems and in vivo. Goldman (12), using mouse L cells in culture, showed reversal of inhibition of DNA synthesis by removal of extracellular drug, despite prior apparent saturation of "high affinity sites." Residual free methotrexate was clearly inhibitory at levels of ≥ 0.12 μM, and there appeared to be correspondingly greater inhibition with higher levels of free drug. That a requirement for free drug existed in vivo was suggested by experiments in which a folate-cleaving enzyme, carboxypeptidase G_1, was able to rescue mice by hydrolysis of residual extracellular drug given 24 hours after administration of an otherwise lethal dose of methotrexate (13). These experiments indicated the importance of two factors, free extracellular drug and, by inference, the duration of tissue

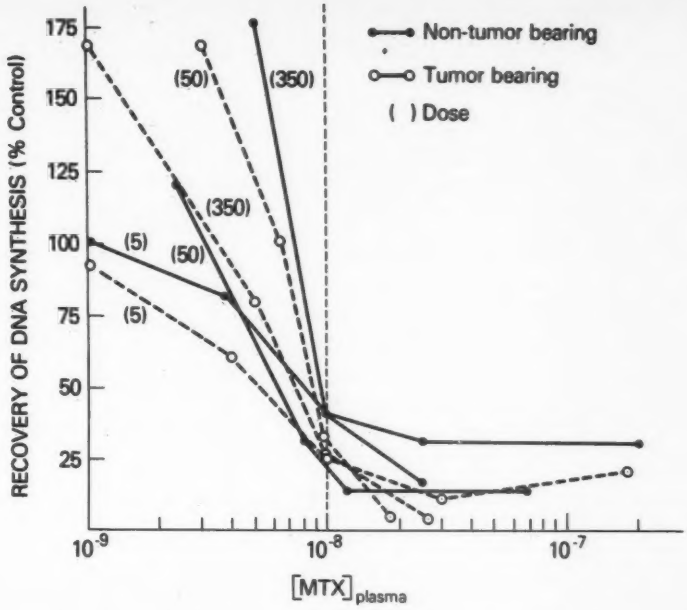

FIGURE 2.—Recovery of ^{3}H-deoxyuridine incorporation into DNA in mouse bone marrow as a function of plasma-methotrexate (MTX) concentration. Doses of drug administered are indicated in parentheses and ranged from 5 to 350 mg/kg in both L1210 tumor-bearing and non-tumor-bearing CDF_1 mice.

exposure, in determining the toxicity of this agent. More recent work by Sirotnak and Donsbach (14) has shown that intracellular levels of methotrexate at least equivalent to dihydrofolate reductase levels were required to detect partial inhibition of DNA synthesis in L1210 cells in vivo; they found that the in vitro requirement of 10^{-6} M methotrexate for 50% inhibition of deoxyuridine incorporation was considerably higher than needed in vivo to produce equivalent effects. In intestinal mucosa, they again found that drug levels exceeding the concentration of enzyme were required for maximal inhibition of DNA synthesis, and attributed this requirement to the nonstoichiometric titration of enzyme by inhibitor (15).

This experimental work suggested that extracellular drug levels, which are in rapid exchange with unbound intracellular drug, might be monitored as an indicator of free intracellular drug, and thus might have a useful relationship to intracellular drug effect. While the relationship between extracellular and intracellular drug concentration has been determined for several experimental tumors (16) and for intestinal mucosa (15), it is still to be worked out for most tissues.

Initial efforts have been directed at establishing the lowest toxic level of free extracellular drug, a level which would correspond to the minimum inhibitory concentration used in bacteriology. While tissue culture experiments suggested that levels as high as 10^{-6} M were required to inhibit DNA synthesis (12), the lower concentration (10^{-8} M) appears to be the threshold required for suppression of DNA synthesis in vivo in several tissues (17) including mouse and human bone marrow (fig 2). This conclusion was reached in pulse-dose experiments which correlated plasma levels of methotrexate with the incorporation of ^{3}H-deoxyuridine into DNA in the target tissue. Somewhat lower concentrations (5×10^{-9} M) were required to inhibit DNA synthesis in gastrointestinal mucosa. Constant infusion devices were used by Zaharko and co-workers (18) to set plasma methotrexate at specific levels, and these experiments provided unambiguous confirmation of partial inhibition of DNA synthesis in bone marrow at levels of 2×10^{-8} M, and a more complete inhibition of intestinal mucosa at this level. An important common finding of the foregoing studies and the reports of Sirotnak and Donsbach (14,15) was that the target tissues (bone marrow, intestinal mucosa, and various tumor cells) varied in their sensitivity to specific extracellular concentrations of methotrexate. Zaharko et al (18) suggested that these differences in sensitivity could be attributed

to differences in the "permeability" of the tissues to methotrexate. Sirotnak clearly demonstrated differences in the persistence of free methotrexate in the more sensitive L1210 cells compared to intestinal mucosa after pulse doses of drug. As yet, the reason for differential drug persistence in the various target tissues is not understood, although most investigators agree that differences in the transport mechanisms of these tissues must be responsible. While the experiments in whole animals in general agree with the results in tissue culture, the requirement for high concentration of methotrexate (10^{-6} M) to inhibit DNA synthesis in mouse L cells in tissue culture has prompted Goldman (12) to postulate the existence of a second "low affinity" site of dihydrofolate reduction.

There are some difficulties in accepting a strictly quantitative interpretation of the foregoing studies, all of which have utilized ^{3}H-deoxyuridine incorporation into DNA as the basis for assessing the inhibitory effects of methotrexate on DNA synthesis. Agents such as methotrexate, 5-fluorouracil, and 5-fluorodeoxyuridine which inhibit the synthesis of thymidylate, lead to marked expansion of the deoxyuridine-5'-monophosphate (dUMP) pool, into which the labeled nucleoside, ^{3}H-deoxyuridine, must be diluted before conversion to deoxythymidine-5'-monophosphate (dTMP) and incorporation into DNA. Expansion of the dUMP pool was monitored following 5-fluorouracil and ^{3}H-deoxyuridine incorporation corrected in a recent study by Myers et al (19), but this correction has not been made in the methotrexate studies. This type of correction would improve the quantitative reliability of methotrexate studies, particularly in the recovery phase when dUMP pool expansion is likely to be the greatest. Other factors could potentially affect the accuracy of deoxyuridine incorporation including: (*a*) changes in thymidylate synthetase activity, (*b*) change in the dTMP pool due to increased or decreased utilization of thymidine via the salvage pathway, or (*c*) changes in substrate other than thymidine-5'-triphosphate. These factors have not been taken into account in the foregoing studies, but are probably of limited importance in view of the general agreement of most data as to the "threshold" for methotrexate effect.

The Relationship of Drug Concentration and Duration of Exposure to Cytotoxicity

The concept of a "threshold" level of free methotrexate for inhibiting DNA synthesis allowed the first meaningful interpretation of methotrexate pharmacokinetic data. Thus, it was possible for the first time to predict that given levels of drug would be inhibitory to bone marrow or other tissue. Second, it was possible to define resistance to methotrexate in terms of an elevated threshold of sensitivity to the drug, as was observed for human ovarian cancer by Young et al (20). However, the usefulness of the threshold was limited because it provided no information regarding the relative importance of two factors, drug concentration (above the threshold level) and duration of exposure, in determining cytotoxicity. Recent studies in our laboratory in collaboration with Zaharko and Bull (5) have yielded insight into the determinants of cytotoxicity for mouse bone marrow.

In these studies, male C57BL mice received a constant infusion of methotrexate and achieved constant blood levels in a range from 1×10^{-8} to 1×10^{-5} M. At regular time intervals from 12 to 72 hours after beginning the infusion, groups of animals were killed and femoral bone marrow was removed for cell counting and in vitro cloning in a semi-solid methyl cellulose medium. The number of nucleated cells per femur fell to a nadir 30% of control for all drug concentrations $> 10^{-8}$ M, but the nadir was reached most rapidly at highest concentrations of drug (ie, within 12 hours at 10^{-5} M, 24 hours at 2×10^{-6} M, 48 hours at 2×10^{-7} M [fig 3], and approximately 72 hours at 5×10^{-8} M). The number of myeloid colony-forming units (CFU-C) decreased in parallel to the fall in total nucleated cells during the first 24 hours of infusion, but an increase in the percentage of CFU-Cs per 7.5×10^{4} nucleated cells plated was seen during the second 24 hours of infusion at all plasma concentrations, and an absolute increase in the number of CFU-Cs per femur was seen at levels between 2×10^{-6} and 10^{-5} M. Although the increased fraction of CFU-Cs at 10^{-7} and 5×10^{-8} M could be attributed either to recruitment or to selective depletion of non-colony-forming cells, the absolute increase in CFU-Cs at higher plasma levels constitutes strong evidence for recruitment of precursor cells to myeloid colony-forming potential in response to the cytotoxic agent (21). This recruitment of CFU-Cs probably contributes to the great burst in DNA synthetic activity, far exceeding control rates, which follows recovery from single doses of drug (17).

There is evidence that recruitment of CFU-Cs also occurs after single doses of methotrexate as indicated by the work of Vogler et al (22). In those experiments recruitment started 24 hours after drug administration at a time when little free drug remained, while recruitment during constant high-dose infusion also started between 24 and 48 hours despite continued infusion of drug. Vogler further showed that a knowledge of the pattern of recruit-

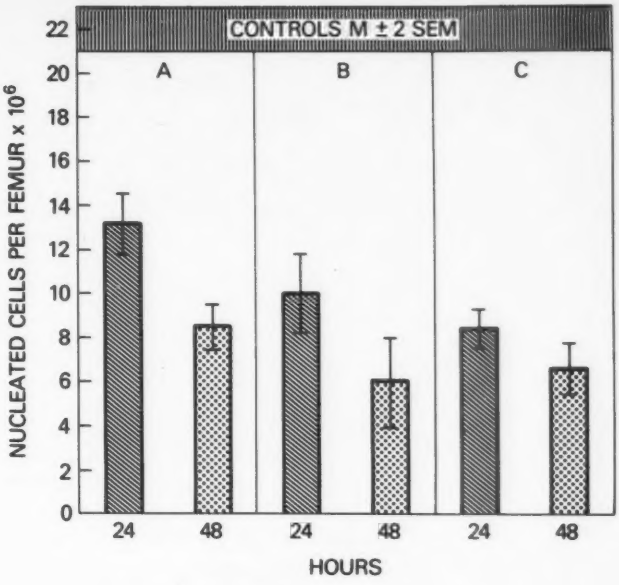

FIGURE 3.—Nucleated cell depletion during 24 and 48 hrs of constant methotrexate infusion. Comparison of the effects of different plasma plateau concentrations (A, 10^{-8} to 10^{-7} M; B, 10^{-7} to 10^{-6} M; C, 10^{-6} to 10^{-5} M).

ment might be of critical importance in the timing of a repeat dose of chemotherapy.

Previous studies in mice (23) using the spleen-colony assay, in which multiple doses of methotrexate were given daily for 72 hours, also demonstrated a self-limiting toxicity to the hematopoietic stem cells (CFU-S), as observed in the current experiments with CFU-Cs.

To summarize, these experiments with mouse bone marrow clearly demonstrate that the rate of cell-kill is greatest for highest drug concentrations, and that cell depletion is a relatively constant function of time until the nadir of cell-kill is reached. This conclusion correlates well with the empiric observations that small doses of drug, given over long periods of time, are equally as toxic as high single doses of drug (24). The recruitment phenomenon probably occurs with other cytotoxic agents and tends to mitigate the effects of single exposures to myelotoxic agents.

These data provide an opportunity to evaluate the concept that the biologic effects of given schedules of drug administration are a function of concentration × time (C × t) (25). In this study, as shown in table 1, the C × t values for four schedules, all of which produce 70% depletion of nucleated cells from marrow, are markedly different, indicating that biologic effect is not a simple function of C × t. Thus, C × t value does not bear a constant relationship to the biologic effect of different schedules of methotrexate administration.

TABLE 1.—Comparison of C × t values for equitoxic schedules

Plasma methotrexate (M)	Time of nadir marrow nucleated cell count (hrs)	C × t at nadir (μ mols/liter × hr)
5×10^{-8}	72	3.6
2×10^{-7}	48	9.6
2×10^{-6}	24	48.0
10^{-5}	12	120.0

Effect of the Physiologic Environment on Methotrexate Activity

It would be appropriate at this point to mention several additional aspects of the cellular environment which affect the degree of biologic activity of methotrexate. These factors include the folate cofactor level of the cell, which is probably of importance,

TABLE 2.—Methotrexate inhibition of CFU-C proliferation and the rescue by nucleosides

Additions to bone marrow culture (M)	Colonies (% of control)
None	100
Methotrexate, 10^{-8}	50
Methotrexate, $\geq 10^{-7}$	0
Methotrexate, $\geq 10^{-7}$ + thymidine, 10^{-5}	0
Methotrexate, $\geq 10^{-7}$ + thymidine, 10^{-6} + hypoxanthine, 10^{-6}	20
Methotrexate, $\geq 10^{-7}$ + thymidine, 10^{-5} + hypoxanthine, 10^{-5}	100
Thymidine, 10^{-4}	20
Thymidine, 10^{-3}	0

although the level of endogenous folates prior to treatment has not been shown to influence methotrexate sensitivity. Nixon et al (11) have demonstrated a build-up of dihydrofolate in L1210 cells after exposure to methotrexate, and this increase in competitive substrate seems likely to play a role in reversing the inhibition of dihydrofolate reductase in a manner analogous to the reversal of fdUMP inhibition of thymidylate synthetase by the physiologic substrate dUMP. A second type of reversal has been shown to occur with purine and pyrimidine nucleosides, the end products of folate metabolism. A combination of thymidine and either hypoxanthine or adenosine has been required in most in vitro systems for full reversal, although purine deprivation appears to play a more important role in cytotoxicity in L5178Y cells (26) than in other cells (27, 28).

Tattersall et al (29) have attempted to improve the therapeutic index of methotrexate with multiple pulse doses of thymidine given every 8 hours for 3 of 4 days after the antifolate. The improvement in therapeutic results seen with combined methotrexate and thymidine was attributed to preferential rescue of normal tissues by thymidine, with continued cytotoxicity for tumor cells due to lack of purines. This interpretation is difficult to reconcile with experiments in tissue culture in which it was clear that both a purine nucleoside and thymidine were required for rescue of mouse bone marrow (table 2); thymidine alone had no rescue effect on the dose/cell-kill curve of methotrexate. The combination of 10^{-5} M thymidine and 10^{-5} M purine nucleoside noncompetitively prevented methotrexate toxicity to bone marrow in culture, but higher concentrations of thymidine alone, or in combination with purines, resulted in toxicity to the CFU in vitro. It is possible that the levels of purine nucleosides circulating in the mouse might be sufficient to allow rescue if supplemented with exogenous thymidine, as in the Tattersall experiment. Little is known, at present, concerning the concentration of circulating nucleosides in man or experimental animals, or the changes in these levels that take place during cytotoxic chemotherapy. In view of the experiments discussed above, one must admit that the relationship between drug level and biologic effect could be altered by changes in nucleoside levels, and this may prove a fruitful area for further investigation.

To summarize, this paper has dealt with our present understanding of the determinants of methotrexate cytotoxicity, specifically the role of free-drug concentration, duration of exposure, and the physiologic environment, including folate and nucleoside pools. In order to provide a rational basis for chemotherapy in man, additional information is needed regarding these factors and their relationship to cytotoxicity for normal and malignant tissues in man.

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Potential Advances in the Clinical Use of Arabinosyl Cytosine[1,2]

Dah Hsi Wang Ho[3,*]

SUMMARY

Arabinosyl cytosine (ara-C) is active in the treatment of patients with acute myelogenous leukemia (AML). Because of its rapid inactivation to arabinosyl uracil (ara-U), ara-C, a phase-specific agent, is administered by prolonged iv infusion. This regimen sustains an effective plasma drug level for at least twice as long as the leukemic cell generation time. The Alza Corp, Palo Alto, Calif, developed a portable liquid infusion system which provides an adjustable infusion rate independent of patient position. Clinical pharmacology shows that patients receiving continuous ara-C at therapeutic doses via the liquid infusion system achieved an effective and constant plasma ara-C level. Cyclocytidine, a water-soluble ara-C depot form, may replace the ara-C continuous iv administration. In man, after a single iv injection of cyclocytidine, the plasma disappearance curve of ara-C derived from cyclocytidine is curvilinear. The half-life of this ara-C, estimated from the terminal phase, is 8 hours, much longer than that of ara-C by direct ara-C administration. When administered im or sc, cyclocytidine is rapidly absorbed. One hour after the drug administration the ara-C level hydrolyzed from cyclocytidine reaches a maximum, and then declines at a rate similar to that of the iv route. This suggests that all three parenteral routes should be equally effective, although for many practical reasons, the sc route is the route of choice for administering cyclocytidine. The use of a deaminase inhibitor, tetrahydrouridine, not only decreases the ara-U formation, but also increases the ara-C uptake in AML cells in vitro. This is largely due to the increase in ara-C triphosphate which is not observed in human normal marrows. This combination may increase the ara-C selectivity against AML. Pharmacologic studies in man will ascertain the clinical value of this approach.

[Cancer Treat Rep 61:717–722, 1977]

Arabinosyl Cytosine (Ara-C)

Ara-C is one of the most active drugs for treating adults with acute myelogenous leukemia (AML) (1,2). It is rapidly inactivated by cytidine deaminase to arabinosyl uracil (ara-U) (3). Twenty-four hours after a single iv injection of tritiated ara-C in man, most of the injected drug (80%) appears in the urine: 72% as ara-U but only 8% as the unchanged drug which is virtually all excreted in the first 4 hours (4). The plasma drug level falls sharply with an initial half-life of 12 minutes, and 111 minutes for the second half-life (fig 1). As a result, the schedule of administration influences the therapeutic index of this phase-specific drug which is much more effective as a 5-day continuous iv infusion (5). This regimen sustains a low, but effective plasma ara-C level for at least twice as long as the generation time of the leukemic cells (fig 2) (4). At equilibrium, the ara-C level in the cerebrospinal fluid (CSF) is 60% that of the plasma (fig 2). This is explained by the fact that there is negligible cytidine deaminase in the CSF or brain parenchyma. A single dose (50

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[2] Arabinosyl cytosine: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

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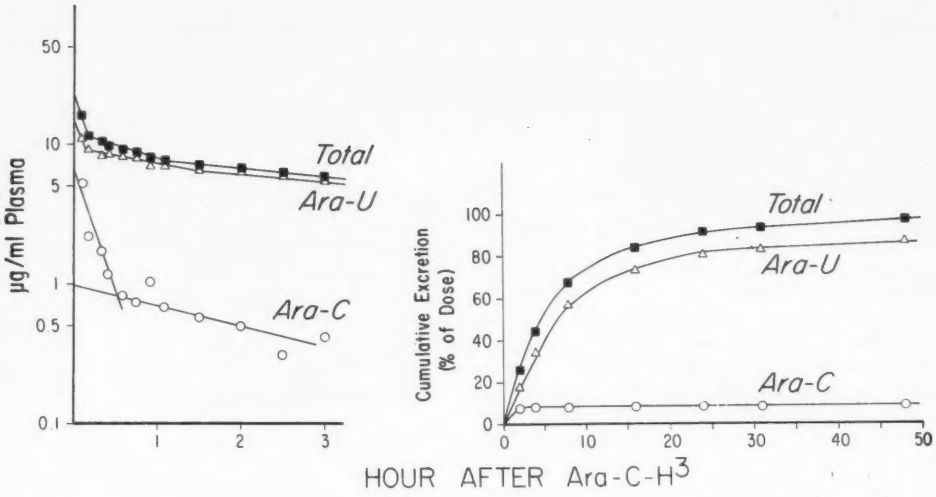

FIGURE 1.—Plasma disappearance curves and cumulative urinary excretion of total radioactivity; ara-C and ara-U in patients after a single iv administration of tritiated ara-C (200 mg/m²).

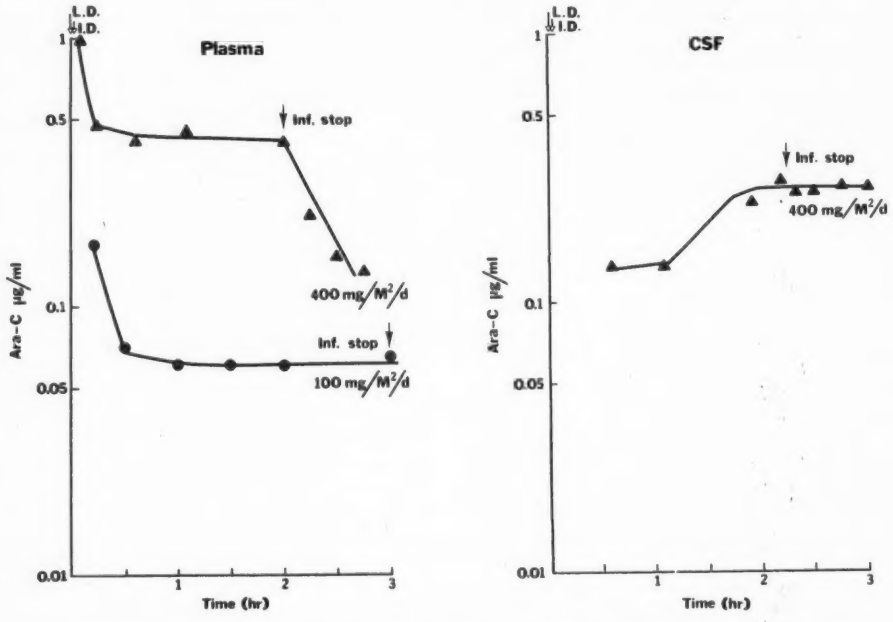

FIGURE 2.—▲—▲ = steady-state plasma and CSF ara-C levels in patients after an iv loading dose (L.D.) of 56 mg followed by a continuous infusion dose (I.D.) of 400 mg/m²/24 hrs. ●—● = steady-state plasma ara-C level in a patient after an iv L.D. of 48 mg followed by a continuous I.D. of 100 mg/m²/24 hrs.

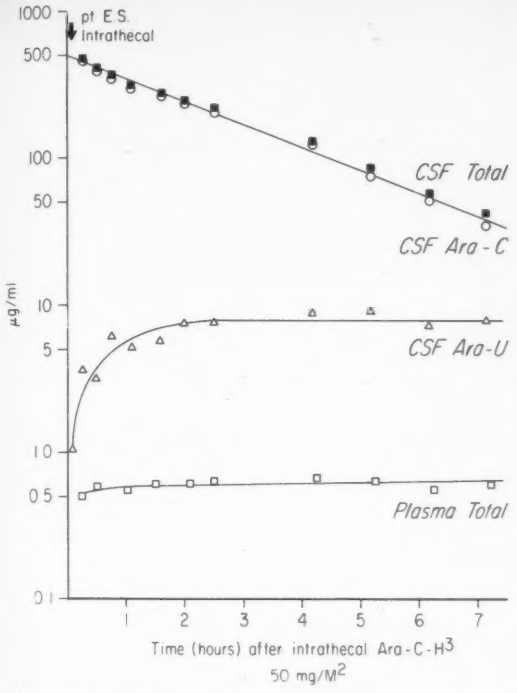

FIGURE 3.—CSF levels of total radioactivity, ara-C and ara-U, and plasma total radioactivity in a patient after an intrathecal injection of ara-C (50 mg/m²). ■—■ = CSF total radioactivity of ara-C and ara-U; ○—○ = CSF radioactivity of ara-C; △—△ = CSF radioactivity of ara-U; □—□ = plasma total radioactivity of ara-C and ara-U.

mg/m²) of ara-C was administered intrathecally to patients, and the half-life of ara-C measured in the CSF is long (2 hours) (fig 3). Assuming continued exponential decrease in ara-C concentration, an effective level would persist for approximately 24 hours. This probably explains the ara-C activity in the treatment of meningeal leukemia.

Ara-C Depot Forms

Depot forms of ara-C may obviate the continuous iv ara-C regimen. Im or oral administration of the insoluble 5'-palmitate or 5'-benzoate ara-C could not provide an effective plasma ara-C level to fulfill this purpose. A more successful approach is to use a water-soluble depot form, the O^2,2-anhydro derivative of ara-C, cyclocytidine.[1] Cyclocytidine itself is not deaminated but is hydrolyzed to ara-C which is subsequently deaminated to ara-U (6,7). The cyclocytidine hydrolysis proceeds at a slow but measurable rate after administering single iv doses in man (8). The plasma disappearance curve of ara-C derived from this single iv dose of cyclocytidine is curvilinear, and the half-life of ara-C estimated from the terminal phase of the curve is 8 hours (fig 4). In contrast, figure 1 describes the plasma decay from the direct administration of ara-C at an equal dose and shows that the initial ara-C level is higher but disappears faster than that of ara-C derived from cyclocytidine. Cyclocytidine may thus serve as a depot form of ara-C. In experimental systems, the antitumor effect of ara-C is greater with prolonged low doses than with intermittent high doses (9). In man, 5-day continuous iv infusion of ara-C at 200 mg/m² is more effective than five single daily injections (5). Continuous iv infusion provides an effective plasma ara-C level of 0.1 µg/ml (4), whereas an equivalent single iv dose of cyclocytidine provides an ara-C level of 0.06 µg/ml for 24 hours (8). To sustain a minimum effective plasma ara-C level for 24 hours, either the daily cyclocytidine dose must exceed 200 mg/m² or the daily doses of 200 mg/m² must be initially preceded by a larger one. This is reflected in the results of the phase I clinical trial; doses of cyclocytidine of 300 mg/m²/day caused mild myelosuppression, similar to that noted after a 5-day continuous iv dose of ara-C of 200 mg/m²/day.

When administered im or sc, cyclocytidine is rapidly absorbed (8). The plasma level of ara-C derived from cyclocytidine reaches its maximum 1 hour after drug administration. It then declines at a rate similar to that of the iv route. In addition, the area under the plasma ara-C disappearance curve, ie, concentration × time, is identical for iv, im, or sc routes of administration (fig 5). This suggests that all three parenteral routes should be equally effective, as further supported by the results of the 24-hour cumulative urinary excretion (fig 6). However, when the drug is given orally, it is incompletely absorbed (fig 6). Therefore, for many practical advantages, sc injection is the route of choice for cyclocytidine administration. A clinical phase I trial using this route revealed toxicity similar to that of an iv injection (8). The phase II trial is now under way.

Liquid Infusion System

A liquid infusion system has been developed by the Alza Corp, and applied to patients receiving continuous iv ara-C. This system is light-weight, portable, and disposable, and provides an adjustable, controlled infusion rate independent of patient position. The clinical pharmacology shows that pa-

[1] Cyclocytidine: NSC-145668; CAS reg. No. 10212-25-6; 6*H*-furo[2',3':4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3a,9a-tetrahydro-3-hydroxy-6-imino-, monohydrochloride.

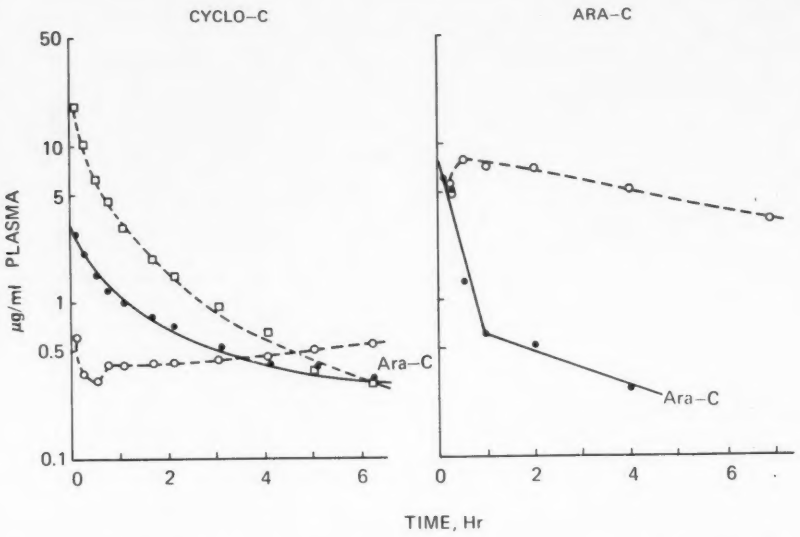

FIGURE 4.—Comparisons of the plasma ara-C levels in patients after a single iv dose (200 mg/m²) of cyclocytidine (cyclo-C) (A) or ara-C (B). □ = cyclo-C; ● = ara-C; ○ = ara-U.

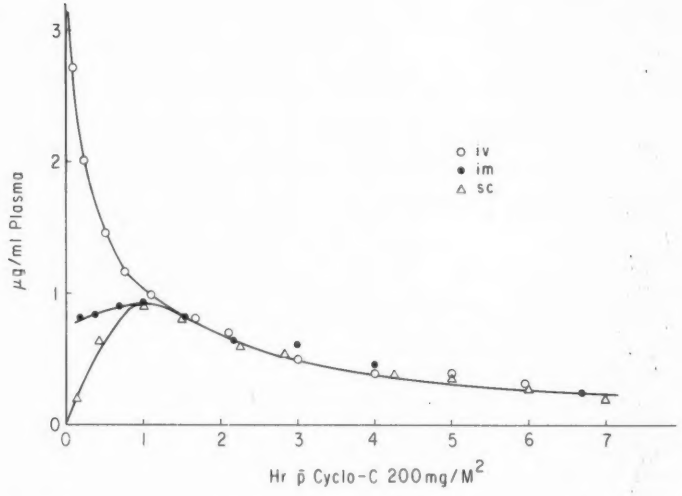

FIGURE 5.—Patients' plasma ara-C disappearance curves derived from iv, im, or sc injections of cyclocytidine (cyclo-C) (200 mg/m²).

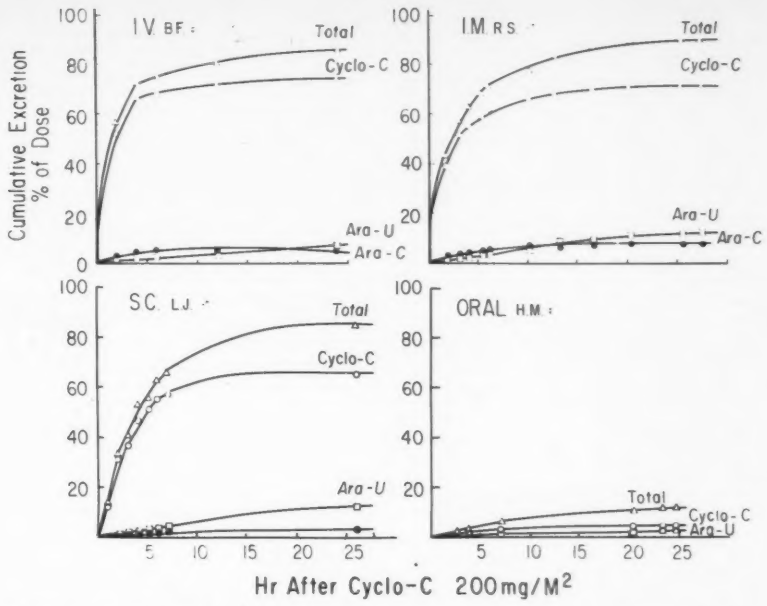

FIGURE 6.—Cumulative urinary excretion of cyclocytidine (cyclo-C) and metabolites, ara-C and ara-U, in patients after iv, im, sc, or oral administration of cyclo-C (200 mg/m²).

tients receiving continuous ara-C at therapeutic doses via the liquid infusion system achieved an effective and constant plasma level. It is a convenient delivery system, particularly for nonhospitalized ambulatory patients.

Predictive System and Resistance

Ara-C is deaminated to an inactive product, ara-U, and is activated by serial kinase reactions to form its triphosphate which inhibits DNA synthesis. The amount of phosphorylated products has been correlated with the response of experimental tumor systems as well as human leukemia to ara-C (10–12). In addition, it has been shown that the ara-C triphosphate level and its duration determine the chemotherapeutic efficacy of ara-C against L1210 leukemia (13). After repetitive exposure of HeLa cells in culture to low levels of ara-C, the cytidine deaminase activity is increased (14). This has also been implicated in human leukemic cells (15). Recent studies, using partially purified enzymes, have shown that the deoxycytidine kinase activity decreases and cytidine deaminase increases with maturation of normal and leukemic granulocytes (16,17). This finding may well explain the current conflicting results on the mechanism of ara-C resistance. If standardized techniques for separating cell fractions, assaying enzyme activities, etc, were used, the mechanism of ara-C resistance might then be ultimately defined, and the predictive system for ara-C nonresponders and for development of resistance could then be assessed.

Tetrahydrouridine[5]

The human liver contains an appreciable amount of cytidine deaminase which is the primary cause of the short plasma half-life of ara-C in man (3,4). A deaminase inhibitor, tetrahydrouridine, not only inhibits the ara-U formation but also increases the ara-C uptake by fourfold in AML cells in vitro (18,19). This is largely due to the increase in ara-C triphosphate. However, such an increase by tetrahydrouridine is not observed with human normal marrows where the phosphorylating activity is low. Ara-C in combination with tetrahydrouridine may increase the ara-C selectivity against AML. Pharmacologic studies of this combination in man will ascertain the clinical value of this approach.

[5] Tetrahydrouridine: NSC-112907; CAS reg. No. 5627-05-4; 2(1*H*)-pyrimidinone, tetrahydro-4-hydroxy-1-β-D-ribofuranosyl-.

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Physiologic Disposition of Cytosine Arabinoside and Its Derivatives in Man [1,2]

W. Kreis,* T. M. Woodcock, M. B. Meyers, L. A. Carlevarini, and I. H. Krakoff [3,4]

SUMMARY

Enzymatic and clinical pharmacologic studies indicate that 2,2′-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine (AAFC) is relatively resistant to enzymatic deamination and is not phosphorylated but slowly releases 1-β-D-arabinofuranosyl-5-fluorocytosine. The latter is deaminated rapidly to 1-β-D-arabinofuranosyl-5-fluorouracil. After iv injection of labeled cytosine arabinoside (Ara-C), 2,2′-anhydro-1-β-D-arabinofuranosylcytosine, and AAFC into patients, radioactivity appears in substantial amounts and persists for a prolonged time in the saliva. AAFC slowly penetrates into the cerebrospinal fluid, reaches significant levels, and is retained there for a long time. Ara-C, due to rapid deamination in plasma, does not provide sustained levels of Ara-C in the cerebrospinal fluid. It is likely that this difference between AAFC and Ara-C is due to reduced polarity of the former compound.

[Cancer Treat Rep 61:723–726, 1977]

From what is known about the behavior of cytosine arabinoside (Ara-C) in mammals, two factors emerge as being crucial for the activity of the compound: (*a*) metabolism of Ara-C to its 5′-di- and -triphosphates is a prerequisite for biologic activity, and (*b*) deamination inactivates Ara-C since the product of deamination, 1-β-D-arabinofuranosyluracil (Ara-U), has not exhibited biologic activity. The study of these enzymes, the kinases and deaminases, their occurrence in different tissues, and their activities upon derivatives of Ara-C iscrucial for further progress in the search for more specific, less toxic, and better drugs of the Ara-C type.

In order to evaluate the first step in the metabolism of Ara-C, its phosphorylation to 1-β-D-arabinofuranosyl-5′-monophosphate, we isolated and highly purified the kinase responsible for the phosphorylation in the 5′ position of deoxycytidine (CdR) and Ara-C (EC 2.7.1.74). As the source for this enzyme, we chose the P815 mouse neoplasm which is sensitive to Ara-C. The purification ultimately led to a homogeneous protein, considered to be pure kinase, as evaluated by several physical methods.

MATERIALS AND METHODS

The isolation and purification of the CdR-kinase will be reported elsewhere.

Deaminase assay.—Each assay tube contained Krebs-Ringer buffer (pH 7.4), 1000 × *g* supernatant from a 20% mouse kidney homogenate (BDF$_1$ mice) or other tissue homogenate (1000 or 10,000 × *g* supernatant), and 4 μmols of cytidine (CR) or Ara-C in a total volume of up to 0.3 ml. After incubation at 37°C, the reaction was terminated by the addition of 200 μl of cold 10% trichloroacetic acid. After removal of protein, the supernatant was added to a Conway microdiffusion dish (Fisher Scientific, Philadelphia, Pa). The amount of ammonia released was determined spectrophotometrically according to Roth et al (1) and Uchida et al (2).

Kinase assay.—The following components were added to each assay tube to a final volume of 0.25

[1]Supported in part by Public Health Service grants CA-08748, CA-15857, and CA-05826 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

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[4]We are indebted to Mrs. Clara S. Gordon for technical assistance.

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ml: adenosine triphosphate, 3 μmols; magnesium chloride, 3 μmols; 3-phosphoglyceric acid, 2.5 μmols; 0.5 M Tris-HCl buffer, pH 8.0, 20 μmols; tetrahydrouridine, 0.15 μmols; substrate, either ^{14}C-labeled 2-CdR (5.1 nmols; specific activity, 1.31 millicuries/mmol) or ^{3}H-Ara-C (5.0 nmols; specific activity, 30 millicuries/mmol) or any other radioactive substrate mentioned in table 1; and 50–100 μl of purified enzyme solution. After incubation for 30 minutes at 37°C, the reaction was stopped by placing the tubes in an ice bath, and duplicate samples of 50 μl were spotted on DEAE filter discs (Whatman DE81, 2.5 cm) and processed as reported earlier (2). The dried filters were placed in diotol (3) and counted in a Tricarb Scintillation Counter Model 3380 (Packard, Downers Grove, Ill).

The methods used for the evaluation of radioactivity in biologic samples have been reported (4).

RESULTS

The best substrate for the purified kinase is CdR (table 1) followed in our extensive series by Ara-C and 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (AAC). The activity of this enzyme clearly is restricted to the phosphorylation of cytosine nucleosides with 2′-deoxy sugar or arabinose moiety. Ara-U is not phosphorylated nor is 2,2′-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine (AAFC), deoxyadenosine (AdR), and deoxyguanosine (GdR).

The discrepancy in the phosphorylation of AAC and the lack of phosphorylation of AAFC is a priori, unexpected, and unexplained, since AAFC is most likely more susceptible to the nonenzymatic ring opening at the 2,2′ position than is AAC, and the resulting products, 1-β-D-arabinofuranosyl-5-fluorocytosine (AFC) and Ara-C, are possibly equally susceptible to phosphorylation. The only possible explanation for the discrepancy can be drawn from the results outlined in table 2. Both AAFC and AFC are strong inhibitors of CdR phosphorylation, whereas AAC does not exhibit such an effect. Tetrahydrouridine activates this enzyme, which parallels the findings of Furner et al (5). Many purine and pyrimidine mono-, di-, and tri-phosphates are inhibitors of CdR phosphorylation also (Meyers M, and Kreis W, unpublished results). It is unlikely that a tissue without an active CdR kinase will respond to Ara-C. According to Kessel et al (6), there is, at least in experimental tumors, a close relationship between the kinase activity and response to Ara-C.

In man, the relationship is complicated by the appearance in many tissues of CR-deaminase. This enzyme clearly counteracts the activity of Ara-C and is thought to be the reason for treatment failures and resistance to Ara-C in human leukemias. Large amounts of CR-deaminase are found in human liver, spleen, and normal bone marrow. Requirements for deamination of CR derivatives include an unsubstituted amino group in position 4 and an unsubstituted OH group or no OH group (such as in CdR) in position 2′ (7,8). The influence of substitution in the 3′ position is insignificant for the deamination of the amino group in the 4 position. Fluor substitution in position 5 significantly increases the deamination of CR, Ara-C, and many of their derivatives. The relative deamination for a series of Ara-C derivatives in comparison to CR is shown in table 3. In an attempt to circumvent the deamination of Ara-C in man, Fox et al (9) synthesized AAFC with the hope that this compound might provide a double-barrel approach to chemotherapy. It was anticipated that AAFC in vivo would slowly release AFC which, in animal experiments, was in some instances superior to Ara-C (10). Deamination of AFC would lead to 1-β-D-arabino-

TABLE 1.—Phosphorylation by purified nucleoside kinase of some Ara-C-related derivatives: reaction at pH 8.0

Substrate	Relative phosphorylation
CdR	1.0
Ara-C	0.1
AAC	~0.1
AAFC	0
Ara-U	0
AdR	0
GdR	0

TABLE 2.—Influence on CdR phosphorylation by Ara-C and Ara-C derivatives (purified kinase system)*

Inhibitors	% change of CdR phosphorylation
CdR + AAC	−4
CdR + AAFC	−43
CdR + AFC	−70
CdR + Ara-C	−42
CdR + AFU	+8
CdR + THU	+29

*Concentration of CdR in assay was 20 μM; concentration of inhibitor or activator was 1 mM.

TABLE 3.—Relative deamination of CR and derivatives in mouse kidney homogenate: reactions at pH 7.4

Substrate	Relative deamination	Remarks
CR	1	
CdR	0.65	
Ara-C	0.75	
AFC	1.08	Also spontaneous deamination
AAFC	0.22	Also spontaneous deamination
AAC	0	

furanosyl-5-fluorouracil (AFU). AFU had antileukemic activity as reported by Yung et al (11).

Clinical pharmacology studies with ^{14}C-AAFC, labeled in the 2 position of the pyrimidine ring, revealed a rapid excretion of the iv administered radioactivity in urine (4). Of four major products found in urine, unchanged AAFC was the main constituent (average, 79%). The next highest product was AFU (average, 12.4%). AFC and 2,2'-anhydro-1-β-D-arabinofuranosyl-5-fluorouracil (AAFU) were found to be only 3.9% and 1.9%, respectively. The time curve of urinary excretion of radioactivity after iv administration of ^{14}C-AAFC is similar to the one observed after administration of ^{3}H-Ara-C (4). The bulk of the material is excreted within the first 4 hours. Oral administration results in slow absorption and slower excretion of the radioactivity in urine. Calculations indicate that about 30% of the orally administered radioactivity is absorbed, which is higher than the absorption after oral administrations of both Ara-C and AAC (12).

After iv administration of 20 mg/kg of AAFC, plasma levels of radioactivity revealed a biphasic pattern with an initial half-life of 0.5–1.5 hours for the first part and 8–24 hours for the second part of the curve. Maximal AFC levels (1 μg/ml plasma) were reached within 1/2 hour and fell to < 0.1 μg/ml only after 2 1/2 hours (4). AFC levels fell more rapidly than did Ara-C levels after iv administration of AAC (12), but more slowly than Ara-C levels after Ara-C injection (13). AFU levels fell less rapidly which might be significant for neoplastic cells responding to AFU (4).

After oral administration of 2 and 20 mg/kg of ^{14}C-AAFC, relatively high plasma levels of radioactivity were maintained for up to 48 hours (4). Analysis revealed a predominance of unchanged AAFC and AFU, whereas AFC and AAFU were identified only in small amounts.

Recently, a strong accumulation of radioactivity was reported in salivary glands after injection of ^{14}C-AAC to mice (14). In a comparison of the excretion of radioactivity in human saliva after injection of ^{3}H-Ara-C (3 mg/kg), ^{14}C-AAC (10 mg/kg), and ^{14}C-AAFC (20 mg/kg), it was found that all three substances or their metabolites were secreted into saliva. The ratios of saliva to plasma radioactivity were comparable in the three studies performed (table 4), although the values in the experiment with ^{3}H-Ara-C- and ^{14}C-AAC-derived radioactivity increased over 4 hours, whereas this ratio attained a peak at 2 hours in the experiment with ^{14}C-AAFC. Qualitative analysis of the saliva revealed, in the study with ^{3}H-Ara-C, the predominant secretion product to be Ara-U in all three saliva samples.

TABLE 4.—Radioactivity in saliva and plasma after iv injection of ^{3}H-Ara-C, ^{14}C-AAC, and ^{14}C-AAFC

Time after injection (hr)	Saliva:plasma ratio		
	^{3}H-Ara-C	^{14}C-AAC	^{14}C-AAFC
1	0.142	0.058	0.053
2	0.148	0.082	0.182
4	0.220	0.094	0.074

The significant and severe complication of leukemia, the meningeal involvement, demands special clinical pharmacology studies. The occurrence of a CR-deaminase in plasma and the complete lack of such an enzyme in cerebrospinal fluid (CSF) make substances such as AAC and AAFC of special interest for chemotherapy trials of central nervous system leukemia. Both substances, due to their decreased polarity and their refractory behavior toward enzymatic deamination, might well be superior to Ara-C for chemotherapy.

When the levels and ratios of AAFC and Ara-C equivalents are compared in human plasma and CSF at different time intervals after iv or oral administrations of ^{14}C-AAFC (table 5), rapid decrease of drug-derived radioactivity is obvious only in the plasma of the iv treated patients. In the CSF, in three of four cases, the levels of radioactivity increased over 24–29 hours, which is well expressed in the AAFC equivalent levels demonstrated in table 5. Qualitative analysis of CSF in one case 48 hours after iv administration of 20 mg/kg of ^{14}C-AAFC revealed only AAFC, indicating little, if any, breakdown of AAFC to AFC. It is likely that relatively high levels of AAFC persisted for extended times.

In a patient receiving ^{3}H-Ara-C iv (3 mg/kg), the short- and long-range plasma versus CSF ratios of Ara-C-derived radioactivity (table 5) fell within the range observed for AAFC. Analysis of the CSF revealed that 46% of the radioactivity was Ara-C and 54% was Ara-U at 70 minutes after injection; 25 hours after injection only 10% was Ara-C and 90% was Ara-U. Frei et al (13) reported maintenance CSF levels amounting to 60% of the plasma Ara-C levels after an equilibrium was achieved by an infusion of 400 mg/m^2/day of Ara-C.

After oral administration of ^{14}C-AAFC, the absorption and penetration into the CSF are much slower but increase over a long time. Analysis of the spinal fluid 24 hours after 2 mg/kg of oral AAFC administration revealed that the amounts of AAFC and AFU in CSF were about the same.

These limited data indicate that unlike Ara-C, AAFC slowly penetrates into the CSF after iv injection, and increases or only slightly decreases in

TABLE 5.—AAFC* and Ara-C equivalents in plasma and CSF of humans evaluated at different times after administration

Dosage (mg/kg)	Route	Time	CSF (μg/ml)	Time	Plasma (μg/ml)	Ratio of plasma: CSF	CSF products
				AAFC			
2	iv	75 mins	0.05	90 mins	0.70	14.0	
		24 hrs	0.01	24 hrs	0.04	4.0	
2	iv	70 mins	0.06	120 mins	1.75	29.2	
		24 hrs	0.48	24 hrs	0.72	1.5	
20	iv	90 mins	0.74	90 mins	18.94	25.6	
20	iv	60 mins	0.52	90 mins	3.64	7.0	
		29 hrs	0.91	24 hrs	0	0	
20	iv	60 mins	1.45	70 mins	14.03	9.7	
20	iv	48 hrs	0.55	48 hrs	0.13	0.2	92% AAFC at 48 hrs
2	orally	24 hrs	0.12	24 hrs	0.18	1.5	50% AAFC and 50% AFU at 24 hrs
20	orally	1 hr	0	50 mins	0.92	∞	
		28 hrs	0.15	24 hrs	0.31	2.1	
				Ara-C			
3	iv	70 mins	0.51	70 mins	3.69	7.2	46% Ara-C and 54% Ara-U at 70 mins
		25 hrs	0.84	25 hrs	0.59	0.7	10% Ara-C and 90% Ara-U at 25 hrs

*Values *from* ref 4.

concentration over 48 hours (and probably even longer times). AAFC seems to release AFC only slowly and probably provides enough AFC to exhibit antitumor activity. Oral administration provides both AAFC and AFU to the bloodstream, and both antimetabolites are transported into the CSF to a limited extent.

Ara-C injected iv provides Ara-C levels in the CSF at 70 minutes after the injection; however, it probably diffuses out of the CSF rapidly while Ara-U equilibrates and predominates at 25 hours after the injection.

While AAFC so far has had no dramatic effect in the treatment of central nervous system leukemias, the clinical pharmacology results reported here indicate improved penetration of AAFC into and its prolonged retention in the CSF; AAFC might be a prolonged source of AFC. Further clinical pharmacology studies and therapeutic trials in meningeal leukemias appear to be justified.

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High-Dose Methotrexate in Acute Lymphocytic Leukemia [1,2]

Arnold I. Freeman,* Jaw J. Wang, and Lucius F. Sinks [3]

SUMMARY

A pharmacokinetic study of methotrexate (MTX) was performed in 60 patients who received high-dose MTX at 500 mg/m² infused over 24 hours. MTX levels reached 1.2×10^{-7} M in the cerebrospinal fluid (CSF) within 10 minutes and remained constant at that level for 24 hours. Forty of these 60 patients were children with acute lymphocytic leukemia. In these 40 patients, simultaneous intrathecal MTX was given along with high-dose MTX. The resultant CSF levels (lumbar area) were higher than those obtained with either high-dose MTX alone or simultaneous intrathecal MTX alone. To date, there have been one systemic and one central nervous system relapse in these 40 children, and the treatment program appears safe to administer.

[Cancer Treat Rep 61:727–731, 1977]

Cranial irradiation plus intrathecal methotrexate (MTX) is standard central nervous system (CNS) "prophylaxis" (1), but cranial irradiation with > 2000 rads followed by iv MTX has led to leukoencephalopathy (2). Approximately 10% of the patients still develop CNS leukemia[4] and cranial irradiation does not protect against other sanctuaries which may be the initial site of relapse. We have been exploring high-dose MTX as a form of both sanctuary and intensification therapy.

This approach was initiated in the Department of Pediatrics at Roswell Park Memorial Institute in 1972 using iv MTX at 500 mg/m² along with concurrent intrathecal MTX which was given soon after bone marrow remission was achieved. Pharmacokinetic studies of MTX in blood and cerebrospinal fluid (CSF) were performed in order to determine whether the addition of this regimen has a pharmacologic basis in preventing CNS leukemia. We are now reporting the clinical and pharmacologic studies in 40 patients so treated.

MATERIAL AND METHODS

Patients

Forty patients with newly diagnosed acute lymphocytic leukemia (ALL) were treated according to the protocol which was instituted in August 1972 (fig 1). Following induction with steroid, vincristine (VCR), and L-asparaginase, three courses of high-dose MTX at three weekly intervals were administered. High-dose MTX was given at 500 mg/m², one third by iv push and two thirds by iv drip over 24 hours. Intrathecal MTX at 12 mg/m² was given from ½ to 2 hours after initiation of iv MTX. Twenty-four hours following completion of iv MTX, a single dose of citrovorum factor was given at 12 mg/m². Following high-dose MTX, the patients received maintenance therapy consisting of daily oral 6-mercaptopurine and weekly oral MTX and pulses of steroid and VCR (fig 1). In addition, 20 patients with ALL who had relapsed on two or more occasions were studied. All these children were studied with simultaneous blood and CSF level determinations when receiving only high-dose MTX.

Eight patients were also studied with simultane-

[1]Supported in part by Public Health Service grant CA-07918 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and by the Association for Research of Childhood Cancer.

[2]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[3]Department of Pediatrics, Roswell Park Memorial Institute, Buffalo, NY.

[4]Acute Leukemia Group B protocol No. 7111: Vincristine/prednisone/dexamethasone (induction) and 6-mercaptopurine/methotrexate/BCNU (intensification) in untreated acute lymphocytic leukemia in patients under 20 years. Activated Feb 5, 1971, and terminated Apr 18, 1974.

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TABLE 1.—Analysis of patient response

Response	No. of patients
Achieved complete remission	40/41
Systemic relapse	1*
CNS relapse	1
Sustained complete remission	38
> 1 yr	20
> 1½ yrs	13
Total No. of patients	41

*High-risk category: age, 15 mos; wbcs 120,000/mm³.

ous blood and CSF level determinations after both high-dose MTX and intrathecal MTX were administered. Intrathecal MTX was given at 12 mg/m² from ½ to 2 hours after the start of high-dose MTX. CSF levels of MTX were also measured in five patients after only intrathecal MTX was administered.

L1210 ascitic tumor resistant to MTX was chosen as a source of dihydrofolate reductase because of its high dihydrofolate reductase activity (3). MTX in serum and CSF was determined by the method of Werkheiser et al (4). The techniques we employed have been described in greater detail (3). High-dose MTX alone was also employed in five children in the treatment of overt CNS leukemia and CSF wbcs were counted before and 3 days after high-dose MTX.

Clinical Studies

To date, among the patients with newly diagnosed ALL, one systemic relapse and one CNS relapse have occurred. The remainder are in continuous sustained remission from 1 to 44 months. Twenty children remain disease-free for > 1 year, 13 for > 1½ years, and three for > 2 years (table 1 and fig 2).

Toxic effects secondary to high-dose MTX were relatively few; vomiting during administration and stomatitis were the most common side effects. Stomatitis appeared more commonly in the teenagers. No leukoencephalopathy was encountered.

In the five patients treated for overt CNS leukemia, three showed a decrease in the CSF wbcs (≥ 50%) by Day 3 as well as an improvement of CNS symptoms. The remaining two patients did not show any response; one of these two was also subsequently resistant to intrathecal MTX therapy.

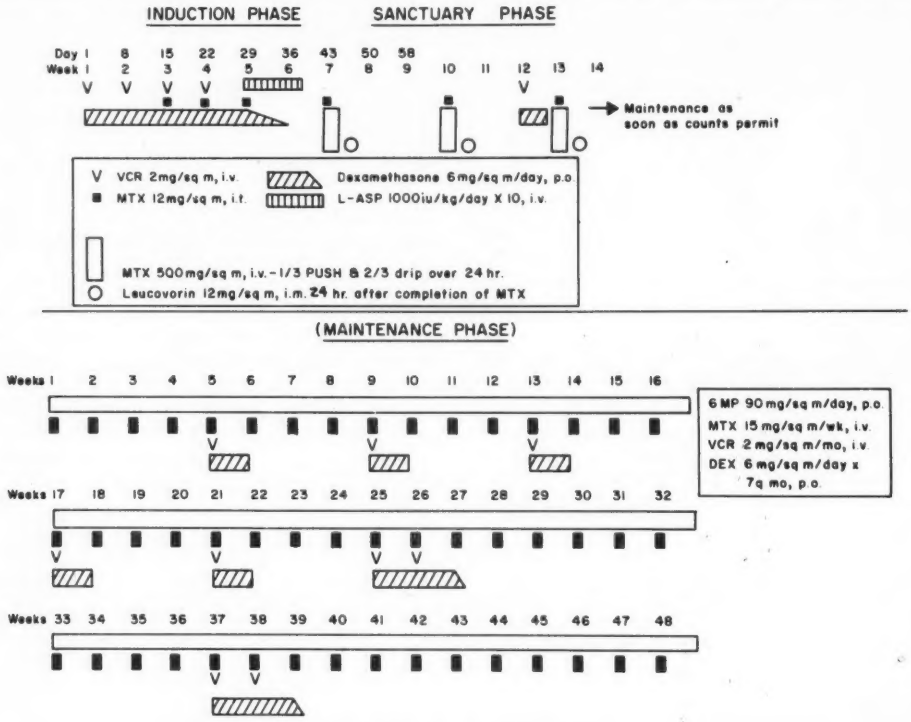
INDUCTION PHASE
SANCTUARY PHASE
Day 1 8 15 22 29 36 43 50 58
Week 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Maintenance as soon as counts permit
V VCR 2mg/sq m, i.v.
MTX 12mg/sq m, i.t.
Dexamethasone 6mg/sq m/day, p.o
L-ASP 1000iu/kg/day X 10, i.v.
MTX 500mg/sq m, i.v.-1/3 PUSH & 2/3 drip over 24 hr.
Leucovorin 12mg/sq m, i.m. 24 hr. after completion of MTX
(MAINTENANCE PHASE)
Weeks 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Weeks 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Weeks 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
6 MP 90 mg/sq m/day, p.o.
MTX 15 mg/sq m/wk, i.v.
VCR 2 mg/sq m/mo, i.v.
DEX 6 mg/sq m/day x 7 q mo, p.o.

FIGURE 1.— Scheme of treatment in ALL.

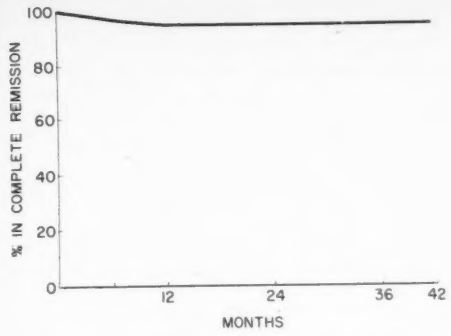

FIGURE 2.—Complete sustained remission duration in 40 patients with ALL.

Pharmacokinetic Studies

Simultaneous CSF and serum MTX levels were obtained from 53 patients who received one or more courses of high-dose MTX. MTX levels in the CSF reached 10^{-7} M within the first 10 minutes and remained constant at this level for 24 hours, during which time the patients were receiving the iv infusions of MTX. Throughout the 24-hour infusion, MTX levels in the blood were approximately 100-fold greater than those in the CSF (ie, 10^{-5} M in serum versus 10^{-7} M in CSF) (fig 3).

Intrathecal MTX alone produced CSF levels $> 10^{-5}$ M in CSF (lumbar area) at 2 hours. By 24 hours, these levels were $< 10^{-6}$ M. However, we do not know what the MTX levels in the ventricles or over the convexity of the brain were, ie, the distribution following intrathecal MTX (fig 4).

When intrathecal MTX was given concurrently with high-dose MTX the resultant levels were 5×10^{-5} M and they were $> 10^{-6}$ M at 24 hours (fig 4), ie, they remained approximately one-half log higher than when intrathecal MTX alone was given.

DISCUSSION

MTX is a potent inhibitor of dihydrofolate reductase. In mice it has been shown that MTX inhibition of DNA synthesis in leukemic cells, bone marrow, and intestinal epithelium depends on a plasma concentration of free (unbound) MTX specific for each tissue (5). Investigation using continuous MTX infusion to achieve constant plasma levels has confirmed that MTX inhibition of DNA synthesis in target tissues is primarily due to the duration of exposure of the tissue to supra-threshold concentrations of drug rather than the peak levels (6).

The clinical application of these findings requires a detailed knowledge of MTX pharmacokinetics in man and a determination of the sensitivity of normal and malignant human tissue in vivo. There is scanty information on the latter. The data of Hryniuk and Bertino suggest that a minimum MTX concentration of 10^{-6} M is needed for therapeutic effect in human cancer cells (7). However, three of our five patients with overt CNS leukemia re-

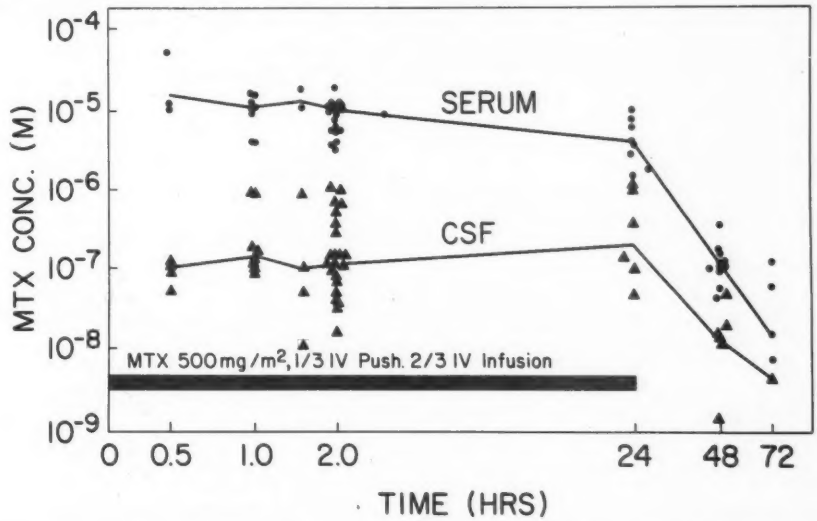

FIGURE 3.—Simultaneous MTX concentration in serum and CSF (lumbar) when MTX was administered at 500 mg/m² iv over 24 hrs.

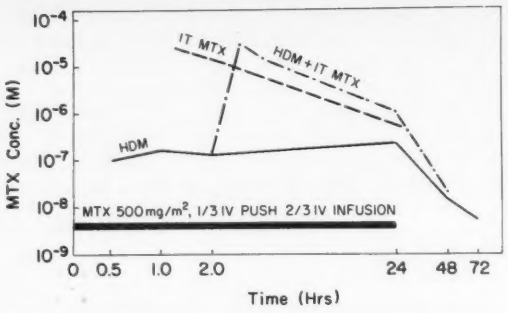

FIGURE 4.—MTX concentrations in lumbar CSF in 60 patients (——) when MTX was administered at 500 mg/m² iv. MTX concentration in lumbar CSF in 5 patients following intrathecal (IT) MTX at 12 mg/m² (---). MTX concentration in lumbar CSF (- - - -) in 8 patients following both high-dose MTX at 500 mg/m² iv plus IT MTX at 12 mg/m². Points on curves are geometric means of the values from the patients.

sponded to MTX concentrations of 10^{-7} M in the CSF. This may be due to the variable individual susceptibility of leukemic cells to MTX concentration or due to the fact that the exposure time to MTX in our patients was longer.

Following an iv bolus of MTX, the plasma level follows a triphasic pattern of disappearance with the half-lives of each phase being 0.8, 3.5, and 27 hours, respectively (8). The third phase with prolonged low levels of MTX is likely the major cause of bone marrow toxicity. Therefore, the toxicity of high-dose infusions can be minimized or prevented if followed within 42 hours by citrovorum factor (9); this may be explained by antagonism of the citrovorum factor to the toxic effects of the slow terminal phase of MTX disappearance in the plasma. From the present data, citrovorum factor given 48 hours after the start of high-dose MTX avoided serious toxicity in all 40 patients with ALL.

Djerassi et al (9) first used high-dose MTX in children with ALL but in a different dose and schedule than the present study, and they observed an effect both in inducing and in maintaining remission.

The rationale in the present protocol for using high-dose MTX with concurrent intrathecal MTX for the prevention of CNS leukemia was based on the concept that by giving high-dose iv MTX and simultaneous intrathecal MTX, higher MTX levels could be achieved than with intrathecal MTX alone; in addition, this would occur in all regions of the CNS, since MTX would not be pumped out of the CNS as quickly as when only intrathecal MTX was given, thus producing more effective CNS prophylaxis. Support for this concept comes from the experiments of Oldendorf and Danson (10) who have clearly shown an increase in C^{14}-sucrose in the rabbit brain following combined iv and ventricular perfusions (10) of this substance. The higher level achieved by combined infusion over either iv or ventricular perfusion alone was believed due to the prevention of C^{14}-sucrose loss from the extracellular space of the brain into blood or into the CSF. Furthermore, following 3 hours of perfusion, C^{14}-sucrose in the brain appeared to be in true equilibrium with both plasma and CSF. Other experiments by Bourke et al (11) have shown that iv infusion along with simultaneous ventricular perfusion of ^{14}C-5-fluorouracil achieved increased levels of this drug in all regions of the brain in the monkey.

The current data indicate that a higher concentration for a longer period was achieved by the simultaneous administration of high-dose MTX with intrathecal MTX over intrathecal MTX alone or high-dose MTX alone. The question of whether intrathecal MTX could be completely avoided by the use of high-dose MTX alone cannot be answered by the present study.

In a recent report, Shapiro et al (12) showed CSF levels of 6×10^{-7} M following iv infusion of MTX at 500 mg/m² over 24 hours given to patients with meningeal leukemia and meningeal carcinomatosis. They also showed negligible ventricular fluid concentrations following a single dose of 50 mg of MTX iv. Also of interest was the fact that MTX given intraventricularly (via an Ommaya reservoir) was distributed rapidly and evenly in the subarachnoid space, whereas MTX given via lumbar puncture showed considerable variation from patient to patient in ventricular fluid concentration.

Whether intrathecal MTX and high-dose MTX can achieve a more even distribution of MTX than intrathecal MTX alone in the CNS of man also awaits confirmation. High-dose iv MTX has the additional theoretic advantage of "hitting" leukemic cells in other pharmacologic sanctuaries such as the testis, ovaries, eyes, spleen, etc, which may be the initial site of relapse (13).

Our preliminary clinical data suggest that this treatment program is safe to administer and to date appears to be effective in the treatment of ALL.

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Treatment of Meningeal Neoplasms[1]

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SUMMARY

Current therapy for patients harboring leptomeningeal neoplasms is reviewed and a new animal model of leptomeningeal neoplasm is described. Intraventricular administration of methotrexate and/or cytosine arabinoside via Ommaya reservoir with or without central nervous system irradiation was used to treat 67 patients (30 with solid tumors, seven with lymphoma, and 30 with leukemia). All of the leukemic patients improved clinically and in 90% the cerebrospinal fluid became normal. Fifty-eight percent of the patients with solid tumors improved; those with breast carcinoma and lymphoma showed the greatest improvement. In about half of the responders, clinical signs of meningeal disease did not recur prior to death from other causes. The frequency of recurrent meningeal leukemia in nine children treated by Ommaya reservoir was reduced to one fourth the incidence occurring in the same children treated by lumbar puncture ($P < 0.05$). Furthermore, median survival from diagnosis for breast carcinoma and lymphoma patients was better than that of patients previously treated by lumbar puncture. At autopsy almost half of the leukemic patients had no meningeal tumor whereas all carcinoma-lymphoma patients did. Device- and drug-related complications were acceptable. We have developed an experimental model of meningeal carcinomatosis by intracisternal inoculation of Walker 256 carcinosarcoma cell suspension into adult Wistar rats. The tumor grows rapidly and is fatal in 13–15 days if 10^6 cells are injected. This histologic pattern closely resembles the diffuse leptomeningeal involvement seen in human disease. Chemotherapy with iv methotrexate was of limited value while intraventricular methotrexate increased survival by 100%. Iv cyclophosphamide was highly effective, increasing survival up to 500% and curing half of the animals. Methyl-CCNU and CCNU increased survival fourfold to eightfold with 50%–70% cures. The experimental model of meningeal carcinomatosis augments the pathologic study of the human disease and for the first time permits testing of potential therapeutic modalities for this increasingly important problem in human solid tumor chemotherapy.

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One aim in establishing this Workshop was to review available and new methods of treating patients harboring neoplasms in their central nervous systems (CNS). Specifically, we were interested in the problem of meningeal tumor spread. Meningeal leukemia became the limiting factor in the otherwise successful therapy for acute lymphatic leukemia in children (1). It was solved first by the therapeutic and later by the prophylactic administration of methotrexate[3] by lumbar puncture (2) and Ommaya reservoir (3) with and without CNS irradiation (4). We are currently faced with what appears to be a similar problem in the treatment of some systemic cancers, that is, the occurrence of meningeal carcinomatosis. For example, a review of 348

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[3]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

autopsies performed at Memorial Sloan-Kettering Cancer Center in 1970 disclosed that 14 patients had meningeal carcinomatosis. Twenty patients were also identified clinically that year. There were also 20 clinical cases of meningeal leukemia. In 1975, five cases of meningeal carcinomatosis had been discovered in the first 70 autopsies and 40 cases had been identified clinically. There were only four cases of meningeal leukemia.

The results of our investigations on the distribution of drugs administered by Ommaya reservoir and by lumbar puncture in patients with meningeal neoplasm have been previously reported (5) and have been reviewed again at this Workshop (6). We have also reported on the use of Ommaya reservoirs in the treatment of meningeal carcinomatosis (7) and this shall be reviewed in addition to a new model of meningeal carcinomatosis in which we have begun chemotherapeutic trials.

Treatment of Leptomeningeal Neoplasm

Ommaya reservoirs have been placed in 82 patients at Memorial Hospital between 1970 and 1974 to treat a variety of conditions (table 1). Reservoirs were placed in 67 patients to treat active meningeal neoplasm and are discussed here. Reservoirs were placed in four leukemic patients for prophylactic drug administration. Eleven patients, indicated as "other diseases," had parenchymal brain tumors, herpes encephalitis, or infectious meningitis.

Of the 67 patients, 30 had solid tumors: 15 breast cancer, seven lung cancer, five melanoma, and three other tumors. Of the other 37 patients, seven had meningeal lymphoma, 22 had leukemia (ten children), and eight had leukolymphoma (who are included with leukemia since the clinical pictures were similar). Adult patients received methotrexate, 6.25 mg/m² twice weekly for several weeks or every 2 days for five doses, and then less frequently. Eleven recent patients, mostly with solid tumors, received methotrexate combined with cytosine arabinoside,[4] 30 mg/m² twice weekly. Treatment of leukemic children was more variable but the pattern was similar. It should be noted that the leukemic children included in this report were those treated prior to the prophylaxis protocol (2) at a time when meningeal leukemia was more common. Additional therapy included focal CNS irradiation, either to brain or spinal cord, in all but four of the patients with solid tumors or lymphoma and in half the patients with leukemia.

[4]Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

TABLE 1.—Ommaya reservoirs placed at Memorial Hospital between 1970 and 1974

	No. of patients given Ommaya reservoir
Active meningeal neoplasms	
Solid tumors	30
Lymphomas	7
Leukemias	22
Leukolymphomas	8
Total	67
Prophylaxis (leukemia)	4
Other diseases	11
Total	82

Clinical improvement was recorded if there was amelioration of neurologic symptoms or signs lasting weeks or months. Of the 30 leukemic patients, only two failed to improve. Diffuse symptoms such as headache, stiff neck, nausea, vomiting, or diminished consciousness were present in two thirds of the patients; these symptoms cleared consistently and often quite dramatically. Focal neurologic deficits also improved, but nine patients were left with some residual deficit such as optic nerve abnormalities or sixth or seventh nerve paresis. Changes in the cerebrospinal fluid (CSF) paralleled clinical improvement, and ventricular fluid returned to normal in 90% of the patients. CNS signs and symptoms eventually recurred in ten of 28 patients, often at the time of the terminal systemic relapse. No patient died solely from neurologic deterioration. All except two of the leukemic patients survived 6 months and half survived 1 year. Autopsies were performed on ten of the 30 patients and, of these, four were free of meningeal tumor.

Of the 26 patients with solid tumors who were adequately treated with two or more intraventricular injections, 15 (58%) improved (table 2). Patients with breast carcinoma and lymphoma had the most improvement. Nine of 14 patients with meningeal breast cancer improved, including three who had no radiation therapy. Half the lung cancer patients improved, and two melanoma patients also had temporary benefit. All six lymphoma patients improved. Focal neurologic signs were present in all patients and were more extensive than the rather isolated findings often seen in the leukemias. Despite improvement, focal signs persisted in all except one patient. Only four patients (or less than one third of those with solid tumors who improved) subsequently developed signs of progressive meningeal tumor, resulting in death. The best median survival among the solid tumors was 5.0 months for

TABLE 2.—Results of intraventricular therapy for solid tumors

Tumor type	No. of patients	No. of patients with—					
		Improvement				No improvement	Inadequate treatment
		Total (%)	Marked	Moderate	Mild		
Solid tumors							
Breast	15	9 (68)	2	5	2	5	1
Lung	7	3	0	0	3	3	1
Melanoma	5	2	0	1	1	1	2
Other	3	1	0	1	0	2	0
Totals	30	15 (58)	2	7	6	11	4
Lymphomas	6	6 (100)	1	4	1	0	0

TABLE 3.—Meningeal leukemia relapses following therapy in 9 patients

	Therapy	
	Intrathecal (lumbar puncture)	Intraventricular (Ommaya)
Total No. of relapses	34	7 (2 questionable)*
Total time (mos) from first treatment	142	112
No. of patient relapses/mo	0.24	0.06 ($P < 0.01$)
Median remission time (mos) from 1st to 2nd episode or death	3.2	4.5

*2 patients without clinical symptoms had cells in CSF compatible with meningeal leukemia.

TABLE 4.—Device-related complications*

Complication	No. of episodes	No. of reservoirs—	
		Revised	Removed
Cannula tip misplaced	3	2	
Cannula tip occluded	6	4	
Infection	7		3
Neurologic deficit	3		
Postoperative pulmonary embolus	1		

*20 episodes occurred in 17 of 110 patients with Ommaya reservoirs.

breast cancer patients and 4.5 months for lymphoma patients. These results are slightly better than those for patients previously treated by lumbar puncture. Thirteen of the patients had postmortem examinations. Meningeal tumor was found in every instance although in two patients given intensive drug treatment, only limited tumor was present.

Nine children with meningeal leukemia were treated first by lumbar injection and later by intraventricular injection (table 3). There were 34 episodes of meningeal leukemia in these nine patients in 142 patient months during lumbar treatment and seven episodes (two of which were equivocal) in 112 patient months during intraventricular treatment. The number of episodes per month in the ventricular treatment period was one fourth that in the lumbar treatment period, a difference that was statistically significant by Chi-square analysis. In these nine patients, the median disease-free interval between the first and second episode while receiving lumbar puncture therapy was 3.2 months, and the median period between the episode preceding the Ommaya placement and the subsequent one, or until death or last followup, was 4.5 months. This difference, while not significant, still gives ventricular administration the advantage. These data indicated that patients with meningeal tumor respond better with drugs given intraventricularly rather than by lumbar puncture.

Complications associated with the device occurred in 17 (15%) of 110 patients (table 4). The cannula tip was misplaced in three patients, and two required revision; the tip became occluded in six, and four of these were revised. Infection by culture developed in seven (only one of whom had clinical meningitis); in three the device was removed but in the other four the device was saved by the administration of oxacillin. Neurologic deficits or transient or persistent focal cerebral abnormalities occurred in three patients, and one patient had a postoperative pulmonary embolism. Two technical factors have lowered the complication rate (8). Since the operative approach has been done transfrontally rather than transoccipitally, no neurologic deficit has occurred. Since intraoperative fluoroscopy has become routine, no misplacement of cannulae has occurred.

Drug-induced complications developed in about 25% of patients at some time during their treat-

FIGURE 1.—Lateral view of brain from patient with malignant melanoma metastatic to the meninges. Pigmentation from melanoma is seen filling interstices of subarachnoid space overlying lateral hemispheres.

ment course (table 5). While fever alone did occur, more often there was accompanying headache, and at times vomiting, or frank meningismus associated with pleocytosis. Florid meningo-encephalitic reactions to methotrexate produced delirium in two patients, both of whom did well with cytosine arabinoside. Mild marrow toxicity occurred in one patient receiving both drugs plus radiotherapy. Two patients experienced leukoencephalopathy of the kind reported with methotrexate therapy after brain irradiation (9).

These preliminary results should be compared with efforts at treating patients with meningeal carcinomatosis 4 or 5 years ago. The literature at that time suggested that the survival of patients with this disease rarely exceeded 6 weeks (10). Our own experience now includes patients who have lived for $> 1\ ^1/_2$ years following a diagnosis of meningeal carcinomatosis and we believe that therapy for the disease is not only possible but that prophylaxis may be desirable as adjunctive treatment for metastatic breast cancer.

Nevertheless, the best route of administration of chemotherapeutic agents is difficult to establish in man. We know for example that when drugs are administered intraventricularly they reach the lumbar subarachnoid space (5), but we do not know if the cerebral subarachnoid space is perfused or if the depth of the sulci, often packed with tumor, receives adequate concentrations of drug. For example, figure 1 shows the brain from a patient with malignant melanoma meningeal carcinomatosis. The depths of the sulci on the lateral surface (fig 1), and especially the subarachnoid spaces at the base of the brain (fig 2), became filled with tumor preventing the passage of CSF. Microscopically the tumor extended deeply within the Virchow-Robin spaces (fig 3) and surrounded the penetrating blood vessels (fig 4). Even over the spinal cord, tumor filled the subarachnoid space with masses; in some patients the spinal cord can become totally encased by tumor. In addition to problems of drug movement, the mechanisms by which such tumor spread produces hydrocephalus and nerve dysfunction have thus far

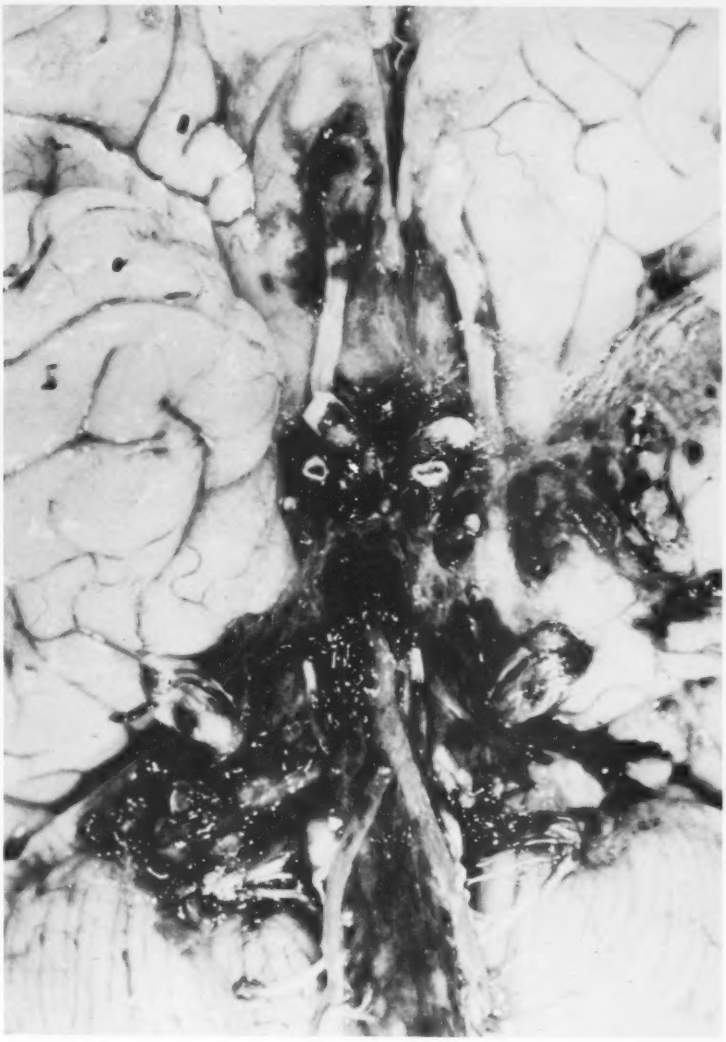

FIGURE 2.—Base view of brain from patient with malignant meningeal melanoma. Tumor fills the interstices of subarachnoid space at base of brain, preventing passage of CSF.

not been investigated. It is possible that there is direct interference by the tumor with the metabolism of the cerebral gray matter and that this causes the cognitive changes which are common in the disorder. Investigating such problems in patients is almost impossible and to undertake such studies, we have established a new model of meningeal carcinomatosis in the laboratory.

FIGURE 3.—Photomicrograph of brain of patient in figs 1 and 2. Tumor invades deeply into Virchow-Robin space. Hematoxylin and eosin, × 25.

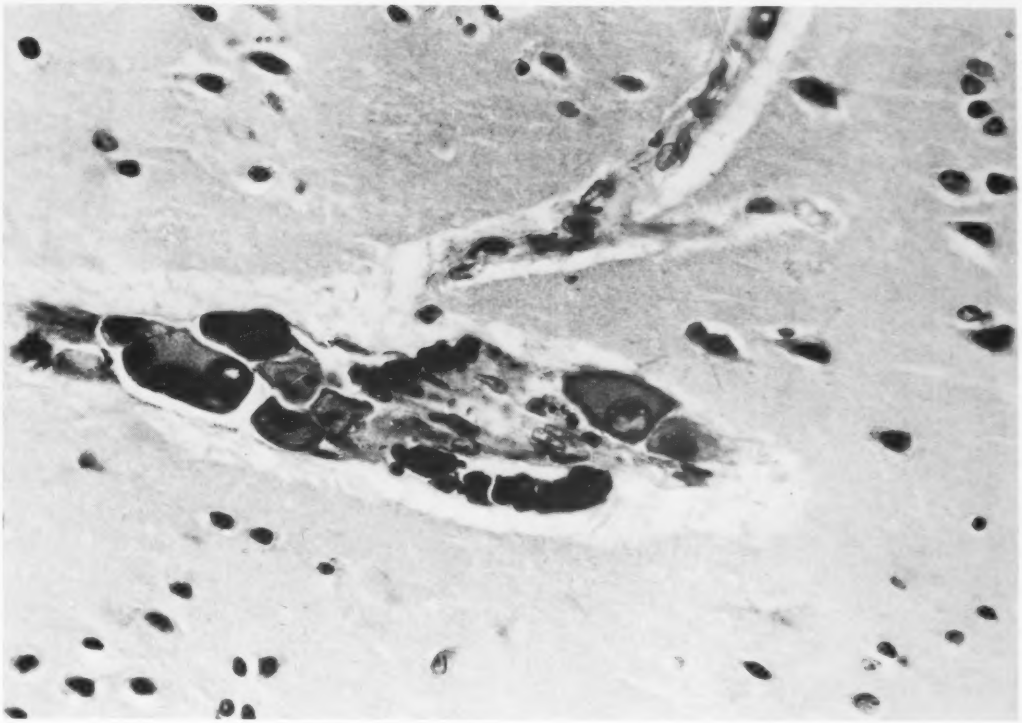

FIGURE 4.—Photomicrograph of parenchyma of brain of patient in figs 1 and 2. Tumor cells are seen surrounding blood vessels within parenchyma of brain. Hematoxylin and eosin, × 250.

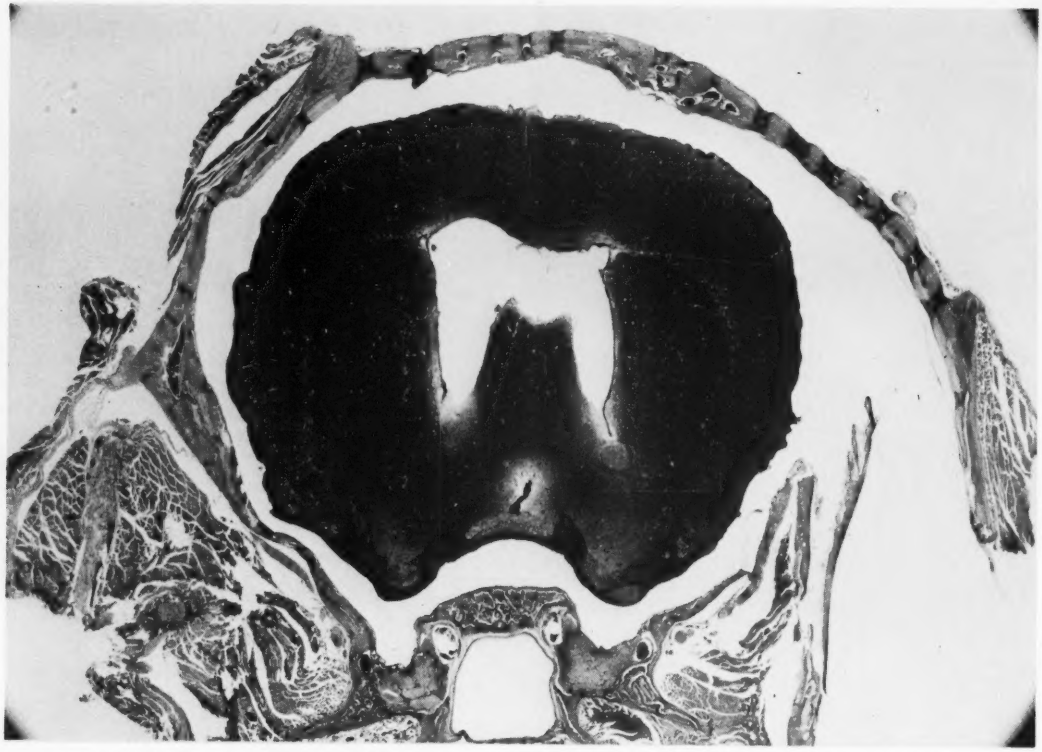

FIGURE 5.—Coronal section of a rat brain injected intracisternally with Walker 256 carcinosarcoma. Tumor has invaded leptomeninges at base of brain and along dorsal-lateral surface. Hydrocephalus is evident. Hematoxylin and eosin, × 2.5.

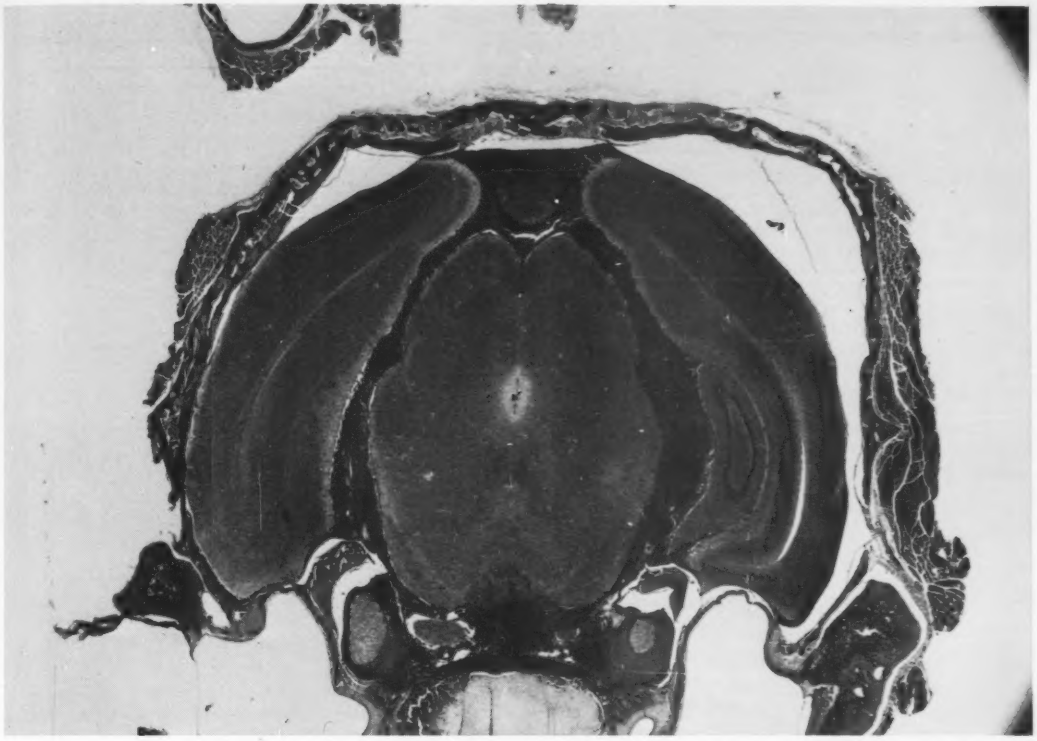

FIGURE 6.—Section through midbrain of rat with meningeal carcinomatosis. Tumor invades ambient cistern and quadrigeminal cistern, and encases midbrain. Hematoxylin and eosin, × 2.5.

A Model of Meningeal Carcinomatosis in the Rat

The model consists of the injection of Walker 256 carcinosarcoma cells (obtained from the Sloan-Kettering Institute Tumor Testing Core Laboratory, New York, NY) into the cisterna magna of 150-g (weight) Wistar rats (Charles River Breeding Farm, North Wilmington, Mass) (11). The tumor cells are inoculated in concentrations of 1×10^4 to 2×10^6 viable cells. Tumor-take occurs in 100% of the animals receiving $> 1 \times 10^5$ viable cells. About 10 days before death the rats start losing weight and then develop neurologic signs consisting of generalized weakness followed by hind limb paralysis, nystagmus, and convulsive twitching of the limbs in some animals. The survival time varies inversely with the size of the inoculum; the median survival time of rats receiving 1×10^6 cells was about 15 days. The histopathologic pattern observed is similar to that seen in human meningeal carcinomatosis (10). Figure 5 shows a coronal section of the rat brain in which diffuse leptomeningeal involvement by tumor at the base and in the coronal fissure is evident; hydrocephalus is also a common feature. Tumor invades the ambient cistern and quadrigeminal cistern encasing the midbrain (fig 6). The tumor also infiltrates along the spinal cord and nerve roots, and the dorsal root ganglia are also invaded. This pattern of diffuse leptomeningeal involvement by

TABLE 5.—Complications of drug treatment

Complication	No. of patients
Fever alone	2
Fever + headache + nausea and vomiting + meningismus	12
Delirium	2
Hematologic	1
Leukoencephalopathy	2
Total	19

TABLE 6.—Iv and intraventricular methotrexate against Walker 256 meningeal carcinomatosis in rats

Route	Dose (mg/kg)	Regimen	Median day of death (T/C)	Median lifespan (% control)	Wilcoxon test significance*	60-day survivors/total No. of rats
Iv	10	Day 5	12/13	92	NS	0/7
		Days 5, 7, 10, and 13	15/15.5	97	NS	0/8
	100	Day 5	16/13	123	NS	0/7
		Day 8	29.3/19	155	$P < 0.05$	1/8
		Day 8	22/13	169	$P < 0.01$	2/7
		Day 11	17/19	89	Lethal	0/8
Intraventricular	1	Day 5	16/14	114	NS	1/9
		Day 8	21/14	150	NS	0/8
	10	Day 5	21/17	124	$P < 0.05$	1/8
		Day 5	17/14	121	NS	0/8
		Day 8	25/20.5	122	NS	2/8
	1	Infusion on Days 5, 8, 12, and 15	26/13	200	$P < 0.005$	0/9

*NS = not significant.

TABLE 7.—Cyclophosphamide, methyl-CCNU, and CCNU (iv) against Walker 256 meningeal carcinomatosis in rats

Drug	Dose (mg/kg)	Regimen	Median day of death (T/C)	Median lifespan (% control)	Wilcoxon test significance	60-day survivors/ total No. of rats
Cyclophosphamide	100	Day 5	>81/13	>623	$P < 0.0005$	5/10
		Day 8	73/14	521	$P < 0.005$	5/9
		Day 8	>94/19	>495	$P < 0.005$	5/8
		Day 11	36/14	257	$P < 0.05$	2/8
Methyl-CCNU	30	Day 5	26/15.5	168	$P < 0.005$	0/10
		Day 5	31/15	207	$P < 0.0005$	2/7
		Day 8	58/17	341	$P < 0.01$	4/9
		Day 8	36/15	240	$P < 0.0005$	1/8
		Day 11	21/14	150	NS	1/7
		Day 11	23/15	153	$P < 0.005$	2/7
CCNU	10	Day 8	29.5/15	197	$P < 0.01$	1/4
		Day 11	23/15	153	$P < 0.05$	1/9
	30	Day 5	>120/14.5	>828	$P < 0.001$	6/7
		Day 5	>120/15	>800	$P < 0.0005$	4/7
		Day 8	38.5/17	226	$P < 0.05$	3/8

tumor cells was observed to be completed 5 days after the inoculation of the tumor.

We have done a series of preliminary chemotherapy investigations in this model (12). These have included iv injections of a number of drugs as well as intraventricular single drug injections and infusions. The chemotherapy results are tabulated in tables 6 and 7. Iv methotrexate had limited effectiveness in the treatment of the disease; 10 mg/kg delivered either once on Day 5 or repeatedly over four doses produced no increase in survival. Methotrexate (100 mg/kg) as a single iv injection produced a maximum increase in survival time of 69% and two animals survived > 60 days. Intraventricular methotrexate in single doses of 1 mg/kg was also ineffective while 10-mg/kg single doses showed minimal effect. A 1-mg/kg intraventricular infusion done over 4 hours on Days 5, 8, 12, and 15 produced a 100% increase in lifespan although there were no long-term survivors. Cytosine arabinoside by intraventricular injection (60 mg/kg, one injection on Days 5, 7, 10, and 13) was ineffective. We also treated this model using iv injections of three drugs: cyclophosphamide, methyl-CCNU, and CCNU. Cyclophosphamide is a highly effective agent against Walker 256 systemically and in this model was

effective in prolonging survival time > 500% with up to half of the animals surviving >60 days. Methyl-CCNU was also effective, increasing lifespan between 50% and 200%. The single most potent drug was CCNU which increased survival 700%–800% and cured over half of the animals.

Thus, in preliminary chemotherapy experiments with this model, intraventricular infusions of low doses of methotrexate were more effective than even large doses administered iv. Far more effective, however, were the nitrosourea drugs which are known to cross the blood-brain barrier and cyclophosphamide whose active metabolite is unknown and may or may not cross the blood-brain barrier. We expect this new model of meningeal carcinomatosis in the rat to furnish further opportunity to investigate new agents and combinations as well as to study the pathophysiology of metastatic meningeal tumor.

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Incidence of Drug-Related Deaths Secondary to High-Dose Methotrexate and Citrovorum Factor Administration [1]

Daniel D. Von Hoff,* John S. Penta, Lee J. Helman, and Milan Slavik [2]

SUMMARY

A review of 498 patients treated with high-dose methotrexate (MTX) with citrovorum factor rescue revealed 29 drug-related deaths. Until the important factors in those deaths are identified, the use of high-dose MTX should be limited to institutions that possess the necessary supportive facilities and the ability to measure serum levels of the drug.

[Cancer Treat Rep 61:745–748, 1977]

The Investigational Drug Branch of the Cancer Therapy Evaluation Program, DCT, NCI, is responsible for monitoring the toxicity and evaluating the effectiveness of the high-dose forms of methotrexate (MTX). Recent encouraging results in the treatment of osteogenic sarcoma using high-dose MTX with citrovorum factor rescue (1,2) have caused an increased clinical interest in the high-dose form of the drug.

Over the past 4 years, several drug-associated deaths have occurred in patients receiving high-dose MTX (3–8). Because of our concern over these fatalities, we have recently undertaken an analysis to determine the incidence of death secondary to high-dose MTX.

MATERIALS AND METHODS

High-dose MTX is defined here as doses > 1 g/m^2 or 20 mg/kg. Currently, the Investigational Drug Branch has 27 approved protocols on file from investigators in the US and Canada for the use of high-dose MTX with citrovorum factor rescue (fig 1). Reports from these investigators on drug-related deaths were reviewed with special attention given to patient characteristics and therapeutic variables.

RESULTS

A total of 498 patients have been treated with the high-dose MTX-citrovorum factor rescue regimen. Most of these patients have been children, with the most common primary diagnosis being osteogenic sarcoma. There have been 29 drug-related fatalities in the 498 treated patients. This represents a 6% overall incidence of drug-related deaths (table 1). Of special interest in table 1 is the wide variation in the percentage of fatal toxicities from investigator to investigator with a range of 0–29%.

The causes of death in the 29 patients have been severe myelosuppression with sepsis or hemorrhage, which caused 80% of patient deaths, and renal failure, which caused 20% of patient deaths.

TABLE 1.—Incidence of drug-related deaths with high-dose MTX

Investigator No.	No. of patients treated	Drug-related deaths	
		No.	%
1	41	0	0
2	150	3	2
3	64	3	5
4	50	2	4
5	46	3	7
6	25	1	4
7	8	0	0
8	15	4	29
9	30	5	17
10	40	5	13
11	6	1	17
12	17	2	12
13	6	0	0
Totals	498	29	6

[1] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.
Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

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FIGURE 1.—Location of investigators using high-dose MTX-citrovorum factor rescue in the US and Canada.

DISCUSSION

A more detailed analysis of the high-dose MTX fatalities is currently being done. Emphasis is being placed on critical parameters, including patient characteristics and therapeutic variables. Figure 2 outlines the patient characteristics presently being considered. We are trying to determine what part age, primary disease, performance status, dehydration, infection, and previous chemotherapy have played in the drug fatalities. Renal function may be an important variable since MTX is excreted largely unchanged in the urine (9,10) and any alteration in renal excretion of the drug could have significant effects on blood levels. The presence of ascites, pleural effusions, or tissue edema after resection of tumor could provide a repository for MTX, causing sustained blood levels of the drug (4,11).

Administration of other drugs concomitantly with MTX may also be an important variable. Since a large percentage of MTX is bound to serum albumin (10), drugs which displace MTX from albumin, such as salicylates or sulfonamides, may increase the drug toxicity. There may also be other drugs administered to the patient which would interfere with the renal tubular secretory mechanisms for MTX or alter urine pH sufficiently to cause a precipitation of MTX in the renal tubules (6,9).

There are several therapeutic variables outlined in figure 3 which we are considering. It is not yet clear whether the high-dose MTX causes more fatalities than lower doses of the drug. The interval of administration may be important because MTX is retained in human tissue for long periods of time (10,11). The mode of administration, bolus or infusion, may influence the toxicity of the drug since infusions have resulted in persistently higher serum levels of MTX (11). Tattersall et al (11) have shown that the measurement of MTX serum levels may be important because patients destined to have myelotoxic effects could be identified by their persistently higher serum levels.

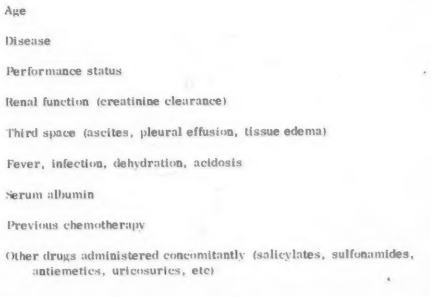

FIGURE 2.—Variables in patients receiving high-dose MTX-citrovorum factor.

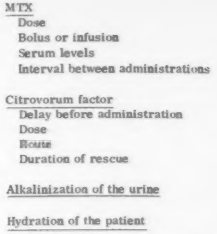

FIGURE 3.—Therapeutic variables in patients receiving high-dose MTX-citrovorum factor.

Citrovorum factor variables include the dose, the delay before its administration, the duration of rescue, and the route of administration. Absorption of oral citrovorum factor in high-dose MTX-treated patients needs to be studied since gastrointestinal mucosal changes induced by high-dose MTX may interfere with absorption of citrovorum factor.

Two final variables include alkalinization of the urine and hydration of the patient. Alkalinization of the urine may be important because MTX precipitates readily when urine pH is < 7.0 (9). Precipitated MTX has been noted in the kidneys of patients dying of renal failure thought to be secondary to MTX (6). This precipitation provides a strong argument for increased urine volume by vigorous patient hydration.

CONCLUSIONS

The overall incidence of drug-related deaths in 498 patients treated with high-dose MTX regimens is 6%. There is a great variation in percentage of drug fatalities from investigator to investigator. Of additional importance is the fact that these deaths have occurred despite care by experienced investigators in major cancer centers.

From the information we now have on hand, it seems that close attention to renal function, adequate hydration of the patient, alkalinization of the urine, and measurement of serum MTX levels are necessary for patient safety. However, other factors may also be important. Until the important factors in these drug-related deaths are identified, the use of high-dose MTX should be limited to institutions that possess the necessary supportive facilities and the ability to measure serum levels of the drug.

Perhaps Dr. Djerassi summed up our conclusions most accurately in a recent publication when he said high-dose MTX-citrovorum factor rescue "is an unreliable ally. One can use it with impunity and elation until, for no apparent reason, it misfires" (12).

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New "Rescue" With Massive Doses of Citrovorum Factor for Potentially Lethal Methotrexate Toxicity [1,2]

Isaac Djerassi,* Jung Sun Kim, Narayan Nayak, Hanrik Ohanissian, Stevan Adler, and Stella Hsieh[3]

A basic assumption in designing the use of delayed citrovorum factor (CF) rescue following high-dose methotrexate (MTX) was the consideration that CF is a coenzyme which does not compete with MTX at any point of their respective mechanisms of action. MTX is a competitive inhibitor of the interaction of folic acid with its reductase while CF is interconvertible with the actual product of this interaction.

Having clinically established that CF can prevent or reduce toxicity due to MTX, even when given after the MTX (1–3), as shown previously by Goldin et al (4) in mice, we saw little need for more than physiologic amounts of CF, regardless of the dose of MTX used. The two agents, after all, do not compete with each other. Indeed, relatively small doses of CF (60 mg/m²/24 hours), arrived at empirically, were usually sufficient to completely prevent the otherwise overwhelming toxicity of > 9000 mg/m² of MTX. More recently, doses of MTX as high as 30,000 mg/m² were well tolerated with the same daily dose of 60 mg/m² of CF given for 3 days (5).

Our observations, and those of others, provided ample and well-reproduced evidence in favor of the theoretic considerations which led us to the currently used dose-differential in the MTX-CF method.

The main difficulty with this method, however, is the unpredictable toxicity. Catastrophic side effects may develop also in *"good-risk"* individuals with normal renal function despite the usually effective dose schedule of CF. Additional CF rescue with the same doses of CF frequently appears to arrest toxicity. Occasionally, however, this is ineffective and death is likely. In such cases, mucosal sloughing with thick choking mucus, total-body skin rash, complete marrow eradication, kidney failure with uremia, and anuria usually lead to respiratory or cardiac failure and death despite the diligent continued administration of 15–20 mg/m² of CF every 6 hours. For some reason, these patients react as if they had never received CF at all.

We have recently centered our attention on the concept that the failure of CF rescue may be due to inadequate penetration of CF into the sensitive normal cells. Unknown endogenous or exogenous conditions may arise and somehow interfere with the active or passive transport of CF into cells. The net result of such occurrence would be the unavailability of the CF administered to the cells saturated with MTX.

This explanation of failing "rescue" resembles in many ways our initial interpretation of clinical ineffectiveness of MTX in resistant leukemia patients (1,6). We attributed such resistance to relatively inadequate binding of intracellular dihydrofolic acid reductase or decreased permeability of the cells to MTX (natural selection by survival of the less permeable cells). The high-dose MTX pulse was devised in order to force more MTX into such cells, according to the principle of concentration × time (C × t) in determining passive diffusion across the cell membrane (1,3,6,7). Tumor cells resistant to smaller doses of MTX were thus affected. If MTX toxicity develops despite the usual CF rescue, CF could have failed to reach the critical normal cells. In a sense, they may have been "resistant" to the dose of CF given.

In searching for means to increase the penetration of CF into these cells, we followed our previous approach with MTX, namely we increased the C (concentration) part of the product C × t. This meant, of course, administration in continuous infusion of massive doses of CF, all with the hope of passive saturation of the critical cells with this agent. Instead of milligrams, we administered grams of CF per day.

For this study, we selected ten patients who developed early and severe MTX-toxic effects, despite receiving the usual rescue with CF. Based on previous experience with such patients, at least one

[1] Supported in part by Public Health Service grant CA-11630 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Citrovorum factor: NSC-3590; CAS reg. No. 6055–86–5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl}-, calcium salt. Supplied as calcium leukovorin by Lederle Laboratories, Pearl River, NY.

Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}.

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TABLE 1.—Massive CF for potentially lethal MTX toxicity

Patient No.	Diagnosis	MTX dose (g)	Day of MTX toxicity	Toxic signs	CF	Status
1	Glioma	50	4	Throat and mouth ulcers, skin rash, and jaundice	3 g/1 day	Improved in 24 hrs; recovered
2	Carcinoma, lung	30	4	Skin rash and throat and mouth ulcers	3 g/1 day	Improved in 36 hrs; cleared in 4 days
3	Carcinoma, lung	18	5	Skin rash, trachial irritation, mucosal ulcers, and jaundice	2 g/1 day	Improved in 48 hrs; recovered
4	Carcinoma, lung	25	5	Skin rash and mouth ulcers	5 g/1 day	Improved in 24 hrs; recovered
5	Hepatoma	12	4	Skin rash and throat and mouth ulcers	1 g/1 day	Improved in 24 hrs; recovered
6	Carcinoma, lung	24	5	Mucosal ulcers	4 g/2 days	Improved in 48 hrs; recovered
7	Carcinoma, colon	6	2	Skin rash and mouth ulcers	8 g/2 days	Improved in 48 hrs; recovered
8	Glioma	24	7	Skin rash and mucosal ulcer	4 g/1 day	Improved in 36 hrs; recovered
9	Lymphosarcoma	3 2	4 5	Skin rash and mucosal ulcer	4 g/2 days	Improved in 48 hrs; recovered
10	Lymphosarcoma	1.2	4	Skin rash	3 g/1 day	Improved in 24 hrs; recovered

half of them would have required intensive care support with > 10% mortality. MTX levels obtained in eight of these patients showed 0.16–7.70 μg/ml of serum in circulation at Day 3 or 4 after MTX. This could possibly explain their toxic reactions in the absence of effective CF rescue. Seven patients received only 1-day infusion of 1–5 g of CF and three others were given 4–8 g for 2 days. As shown in table 1, all ten patients showed arrest of the developing skin and mucosal toxicity within 24 hours and usually started recovering in 48 hours. Bone marrow depression requiring platelet and wbc transfusions did, however, develop in all ten patients. Impairment of renal function was arrested in four patients. Toxic hepatitis was arrested in three patients who recovered fully. There were no MTX-related deaths in the group of ten patients.

The data presented here are preliminary. They were described because of the contrast of the outcome with this group of patients as compared to our previous experience. Since MTX-CF rescue is now rather widely used, especially for osteogenic sarcoma (8), it was felt that this information may be useful to investigators facing catastrophic MTX toxicity.

It should be emphasized that the doses of CF used in this study were selected to offer an excess of the agent, possibly much higher than the dose actually required for the desired effect in individual patients. This was intended to test the concepts discussed above regarding the occasional failure of the standard CF rescue. Further studies are needed to determine the minimal required dose of CF in "CF-resistant" patients. Although, as projected, 1-day "massive rescue" was sufficient in most patients, further work on the dose-schedule aspect of this study is needed. It is very likely that massive rescue will also protect undue amounts of tumor tissue; this, however, is a minor consideration when facing extreme MTX toxicity. Since saturation of the tumor with CF may interfere with the effectiveness of subsequent treatments, we use massive rescue only in extreme cases. Should this new rescue prove consistently effective, its impact on the safety and effectiveness of the high-dose MTX-CF rescue method may be substantial.

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Removal of Methotrexate by Filtration-Adsorption Using Charcoal Filters or by Hemodialysis [1,2]

Isaac Djerassi,* Walter Ciesielka, and Jung Sun Kim [3]

Widespread use of high-dose methotrexate (MTX) followed by citrovorum factor[4] (CF) rescue (1,2) has been hampered by occasional and unpredictable toxicity, especially in patients with impaired renal function. Since MTX is eliminated from the blood by the kidneys, drug persisting in such patients beyond the last dose of CF is most likely responsible for the incidence of severe toxicity.

An important consideration in developing the dose and schedule of CF rescue commonly used was the rapid excretion of iv injected MTX. Since a major portion of the drug is rapidly excreted in the urine and the remaining concentration was known to have only limited antitumor effectiveness, it was felt that CF rescue could be started soon after MTX without interfering with its effect. Blood levels of MTX 36–48 hours after infusion of 300 mg/kg, indicate that < 100 mg of the total dose remains in the body's extracellular fluids. Since this amount of MTX is ineffective in most solid tumors but is sufficient to produce toxicity, its removal at that time is desirable. Persistence of such amounts in the body fluids could only contribute to further toxicity by affecting sensitive normal cells, while contributing very little, if any, further antitumor effect.

The use of an artificial kidney to dialyze out the MTX was considered for this purpose and was used in two patients after treatment with high-dose MTX (table 1).

An alternative approach was pursued using charcoal filters (Becton, Dickinson and Co, Rutherford, NJ) initially developed for removal of barbiturates from overdosed patients (3). These filters could easily be fitted into the system for filtration-leukapheresis (4) developed in this laboratory and used on a daily basis for collection of granulocytes from normal donors for transfusion into leukopenic and infected patients. The system is basically a vein-to-vein extracorporeal circuit. The blood is collected and propulsed mechanically from one vein to another, interposing a filter for specific adsorption and collection of the desired blood element. In this case we replaced the usual leukocyte-collecting filter (nylon filter) with the charcoal filter.

Preliminary in vitro trials in this laboratory had shown that MTX, like barbiturates, adheres to charcoal and could thus be removed from the blood. Optimal perfusion rates were determined and used for four patients with metastatic malignant disease after treatment with high-dose MTX. The efficiency of this procedure is illustrated in table 1.

[1] Supported in part by Public Health Service grant CA-11630 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}-.

[3] Mercy Catholic Medical Center, Philadelphia, Pa.

[4] Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl}-, calcium salt.

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TABLE 1.—Removal of blood MTX with filtration-adsorption vs hemodialysis in patients treated with high-dose MTX

Patient No.	Serum MTX level (μg/ml)— Before procedure	At 1 hr	At 2 hrs	At 3 hrs	At 4 hrs	At 5 hrs	Time of procedure after treatment (hrs)
				Carbon filter			
1	60.00	39.00	36.00	32.00	—	—	12
2	21.30	19.50	16.70	13.50	—	—	43
3	0.42	0.37	0.27	0.19	—	—	41
4	0.18	0.16	0.06	0.01	—	—	62
				Renal dialysis			
1	8.80	5.20	4.70	4.10	4.20	4.00	16
2	213.00	141.00	95.00	86.00	71.00	62.00	4

Serum MTX levels were reduced substantially by both hemodialysis and charcoal filtration. In fact, it appeared that the filtration was more efficient than the hemodialysis when the blood concentrations of MTX were low. At the level of 4.0 μg of MTX/ml of serum, dialysis appeared to be rather ineffective, while filtration at these levels continued to deplete the patient of circulating MTX.

The feasibility of filtering out MTX from recipients of high-dose MTX is established. Considerable technical and logistic problems remain to be solved prior to engaging in a controlled study of the value and safety of this procedure in the management of patients treated with high-dose MTX-CF rescue.

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High-Dose Methotrexate in Malignant Gliomas [1]

William R. Shapiro [2,3,*]

Djerassi et al (1) reported on the use of high-dose methotrexate (MTX)-citrovorum factor rescue in the management of seven patients with brain tumors.[4] Five of the seven patients were children with diagnoses of astrocytoma, brain stem glioma, and ependymoma, and two were adults, one with glioblastoma multiforme and one with a medulloblastoma. The maximum single MTX dose varied from 1000 mg/kg iv in one of the adults to 2200 mg/kg iv in one child. Djerassi reports that six of the seven patients showed neurologic improvement; survival ranged from 7 to 23 months and one patient was alive at 10 years. Rosen et al (2) also reported that high-dose MTX with citrovorum factor rescue was of value in the management of recurrent malignant brain tumors in children. They treated 14 patients, 11 with recurrent medulloblastoma and brain stem glioma, and reported objective (pneumo-encephalogram) and clinical evidence of improvement in six of these.

Most of the patients in these reports were children who were able to tolerate large doses of MTX, and most of the tumors were medulloblastomas in which some sensitivity to MTX has been demonstrated (3). In a trial of MTX in adults with malignant brain tumors we have treated six patients with high-grade malignant gliomas, five of whom were showing evidence of progression. This report presents our preliminary results.

METHODS

MTX was administered by iv infusion over 2 hours. Initially, patients were treated at a dose of 500 mg/m^2 preceded 1–4 hours by vincristine (1 mg/m^2 iv).[5] MTX was dissolved in 250–500 ml of dextrose and water. Twenty-four hours after the beginning of MTX, citrovorum factor was begun at a dose of 50 mg/m^2 iv followed by 5 mg orally every 8 hours for nine doses. The dose of MTX was escalated by 500 mg/m^2 approximately every 3 weeks. It became apparent early that a dose of $\leq$ 1 g/m^2 was not toxic, and dosage escalation was increased more rapidly to every 2 weeks according to the schedule shown in table 1 which also lists the corresponding change in citrovorum factor dosage.

Before initiating MTX therapy, each patient required a creatinine clearance of $\geq$ 50 ml/minute and a normal serum creatinine. The patients were given two sodium bicarbonate tablets, 625 mg, every 4 hours for 36 hours prior to MTX and for 12 hours after MTX infusion began in order to alkalinize the urine. The urine pH was checked frequently and as soon as it exceeded pH 7.0 the MTX infusion was begun. Serum MTX determinations were done as previously described (4) at 24 and 48 hours after beginning MTX infusion, and serum creatinine was checked at 24-hour intervals. Patients were well hydrated, receiving up to 3 liters of fluid iv on the day of the MTX. The patients were followed with regular neurologic examinations and, when possible, with repeated computerized transaxial tomography scans.

Six patients were treated with MTX (table 2). Their ages ranged from 13 to 62 years. All patients had biopsy-confirmed malignant brain tumors: glioblastoma multiforme in five patients and a malignant astroblastoma in a 13-year-old boy. All patients had been treated with up to 6000 rads of whole brain radiation therapy, either in the immediate postoperative period or in the recent past, and some of the patients had been treated with previous chemotherapy.

RESULTS

The results in the treatment of the six patients are tabulated in table 2. Each patient received $\geq$ 1 g/m^2 of MTX. One patient was treated seven times, one patient four times, one patient three times, and three patients two times. The maximum dose of MTX utilized was 21 g/m^2 in patient 1. The maximum total dose given to this patient was 42 g in one

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[1] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[2] Department of Neurology, Memorial Sloan-Kettering Cancer Center, New York, NY.

[3] Dr. Bipin M. Mehta's valuable assistance in MTX determinations is appreciated.

[4] Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

[5] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

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TABLE 1.—High-dose MTX-citrovorum factor rescue escalation*

MTX (g/m^2)	Citrovorum factor: Iv dose (mg/m^2) 24 hrs after starting MTX (schedule)	Citrovorum factor: Oral dose (mg) beginning 30 hrs after starting MTX (schedule)
1	50	5 (q8hrs × 12)
1.5–2	100	10 (q8hrs × 12)
3	100	15 (q8hrs × 12)
7	100 (q12hrs × 4 days)	15 (q8hrs × 12)
14	100 (q8hrs × 4 days)	15 (q8hrs × 16)
21	100 (q6hrs × 4 days)	15 (q8hrs × 20)

*Dosages were increased every 2 wks.

TABLE 2.—Results of high-dose MTX-citrovorum factor rescue treatment in malignant brain tumors

Patient No.	Age (yrs)/ sex	No. of treatments	Maximum MTX dose per treatment: g/m^2	mg/kg	Total g	Maximum toxicity	Neurologic status
1	33/M	7	21	522	42	Severe	Progression
2	45/F	4	7	176	12.6	Mild	Progression
3	13/M	3	14	432	19.6	Mild	Progression
4	39/F	2	7	223	9.8	Minimal	Progression
5	39/F	2	1	28	1.58	None	No change
6	62/M	2	1	27	1.8	Minimal	Progression

treatment. As noted below he suffered severe toxic effects associated with this dose.

The neurologic status in patients 1, 2, and 6 was deteriorating at the time MTX therapy was started. In each instance, treatment was followed by acute neurologic worsening which took the form of transient clouding of mental status for a period of 2–4 days following the MTX infusion. The obtundation occasionally progressed to frank coma, and in patient 1, permanent increased neurologic deficit occurred. In addition, patients 1 and 2 showed progressive enlargement of the brain tumor on computerized transaxial tomography scan.

Patients 3 and 4 had had recent resections of recurrent brain tumor, while patient 5 had undergone her first operation for malignant glioblastoma. During the course of therapy, patients 3 and 4 showed transient deterioration of mental status immediately following each treatment and overall progression in nervous system signs and symptoms during the course of the treatments. Patient 5 withdrew from study after two treatments without evidence of deterioration.

Toxicity varied from none to severe. Except for patient 5, all showed abnormalities in neurologic function for 2–4 days following MTX treatment. This dysfunction consisted of mild mental clouding progressing to stupor and coma in its most severe form. Patients who had major signs frequently had worsening of their signs during the posttreatment period. In patient 1, as noted below, frank cerebral herniation occurred on two occasions following his largest dose of MTX. Some nausea was encountered in all patients. In the more severe reactions, mouth ulceration and mild leukopenia were observed.

Patient 1, who received the largest dose of MTX, suffered a sustained severe toxic reaction following his last dose of MTX (table 3). He had just had a resection for recurrent glioblastoma multiforme. He was treated seven times with progressively increasing doses of MTX (table 2). Except for the first dose of 500 mg/m^2, in which the patient suffered no symptoms, each of the treatments was associated with progressively more serious mental obtundation. Twenty-four hours after receiving a total of 42 g of MTX in the last dose, he became drowsy and developed emesis. Over the next 2 weeks his mental status varied from mild lethargy to frank coma and on two occasions he showed neurologic evidence of cerebral herniation and required increased corticosteroid hormones. Severe mucositis was noted on Day 3 and persisted for 4 days. His blood urea nitrogen (BUN), which initially had been normal at 16 mg/100 ml, rose to 62 mg/100 ml 3 days after treatment and was associated with a progressive fall in the wbc and platelet counts. His serum creatinine rose to a maximum of 3 mg/100 ml on Day 5 following MTX treatment. Unfortunately, MTX levels were not determined until the sixth day following treatment and on that day, the blood level

TABLE 3.—Patient 1: high-dose MTX-citrovorum factor rescue (MTX dose = 21 g/m², 522 mg/kg; total, 42 g)

Day	Clinical status	Serum creatinine (mg/100 ml)	BUN (mg/100 ml)	Wbcs ($\times 10^3$/mm³)	Platelets ($\times 10^3$/mm³)	Serum MTX	Fluid intake (ml/day)
0	Alert	0.9	16	5.3	125		3200
1	Drowsy, emesis	1.1	18	9.5	195		2460
2	Lethargy, emesis			5.0			3320
3	Drowsy, mucositis 3+						3220
4	Lethargy, mucositis 3+		62	4.4	119		3010
5	Lethargy, mucositis 2+	3.0	61	2.6	127		3310
6	Semiconscious, mucositis 1+	2.5	52	3.2	115	1.41×10^{-7} M	4230
7	Lethargy	2.2	52	5.4	101	5.52×10^{-8} M	3450
8	Alert	1.8	40	6.0	107	3.7×10^{-8} M	4650
9	Alert			5.6	80		4300
10	Herniation,* emesis						4380
11	Semiconscious			7.7	83		1715
12	Alert			7.0	98		5750
13	Alert	1.2	16	8.2	111	7.75×10^{-9} M	4490
14	Lethargy*		23	6.7	90		4330
15	Alert	1.2	19				3310
18	Alert			8.3	199		

*Increase in steroids.

was elevated at 1.4×10^{-7} M. The patient was treated with large volumes of fluids iv (2500–5000 ml/day). Over a period of 2 weeks he gradually regained consciousness. His wbcs returned to normal as did his serum creatinine and BUN. This toxic reaction was associated with definite worsening of his neurologic state.

Because of the severity of the toxic response in these patients, we discontinued the MTX study. Patient 6 died 2 weeks after his last dose of MTX. All other patients were given other chemotherapy regimens and at this time are either stable or improving.

DISCUSSION

Our experience with high-dose MTX therapy differs from that of Djerassi and Rosen. Except for one patient, all of our patients were adults. All patients had highly malignant supratentorial glial tumors. During the treatment with high-dose MTX, all patients except one had acute neurologic deterioration associated with each treatment and five of the six patients demonstrated overall progression of disease. The maximum dose given to any patient was 522 mg/kg in patient 1, a dose which was associated with a severe toxic reaction that lasted > 2 weeks. The minimum dose given to the patients was 27–28 mg/kg. On the basis of this short experience, we concluded that high-dose MTX failed to demonstrate benefit in therapy for malignant supratentorial gliomas in adults.

One consideration raised by this experience is whether adequate quantities of drug were administered. Djerassi had given as much as 2200 mg/kg in the treatment of his patients with brain tumors although only children received the very high doses. Nevertheless, he treated one adult patient with a maximum of 400 mg/kg and noted neurologic improvement and survival of > 7 months from diagnosis. In one other patient, an adult with a medulloblastoma, a maximum dose of 1000 mg/kg failed to yield improvement. Experience with adults elsewhere in this center treated with a high-dose MTX regimen for carcinoma of the pancreas showed that doses of 1–2 g/m² were effective (5). We demonstrated that the doses of MTX that were used were toxic to the central nervous system, the gastrointestinal tract, the bone marrow, and the kidneys. Nevertheless, the question must remain in all but one of our patients that increasing the dose of drug might have proved effective.

A more theoretic consideration relates to the observation (6) that brain normally does not contain dihydrofolate reductase. If true, and there is some question about this observation (7), it is possible that malignant brain tumors themselves do not contain dihydrofolate reductase, in which case the tumors might not be responsive to MTX. However, medulloblastomas probably respond to MTX admin-

istered intrathecally (3) and in a high-dose iv regimen (2). We are currently attempting to determine the concentration of dihydrofolate reductase in malignant brain tumors in man.

The degree of toxicity which we witnessed in our brief trial with this regimen should be compared to the relative lack of toxicity afforded by many chemotherapeutic agents currently in use against malignant brain tumors (8). Thus, in our patients treated with nitrosoureas alone (9) or in combination (10), toxicity was usually minimal, so that patients lived at home and often carried on full work activity. In no instance was this possible in the patients undergoing treatment with high-dose MTX. In all instances, hospitalization was required for a period of 4–14 days and the patients were unable to carry on normal activity. Thus, even if increasing doses of MTX are shown to be effective, the quality of the patient's existence might substantially suffer.

Finally, mention should be made of the potential for lethal leukoencephalopathy that might develop in such patients, especially after prior radiation (11). Rosen (personal communication) has had such a patient and it is likely that with more experience further cases will develop.

High-dose MTX remains of questionable value in the treatment of adults with malignant glioma. Its role in the treatment of childhood tumors remains to be determined.

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ANNOUNCEMENTS

Fellowship in Medical Oncology

Applications are being accepted from candidates who are interested in a Joint Collaborative Training Program in Medical Oncology involving major teaching hospitals of Brown University's (Providence, Rhode Island) Program in Medicine, beginning July 1978 and July 1979. This Program offers clinical and research activities in cancer chemotherapy, clinical and biochemical pharmacology, and drug development and tumor immunology, and participates in national cooperative oncology groups to aid in the management of patients with solid tumors and hematologic malignancies seen at many of the hospitals in the Providence area. Prerequisites include 2 years of approved Medical Residency and American Board of Internal Medicine eligibility. Candidates with academic interests are encouraged to apply.

Send informative letter and curriculum vitae to: **Paul Calabresi, MD, Professor and Chairman, Department of Medicine, or F. J. Cummings, MD, Head, Division of Medical Oncology, Department of Medicine, Roger Williams General Hospital, 825 Chalkstone Ave, Providence, RI 02908.**

Seminars in Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be a seminar(s) on "The Chronic Leukemias" on September 8, 1977, organized by the Department of Medicine B. The chairperson is Dr. Joseph Sokal.

There is a $10.00 registration fee for this 1-day seminar. ***For further information contact:*** **Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.**

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